ELSEVIER

Associate Editor: S. T. Mayne

Chemopreventive Agents: Protease Inhibitors

Ann R. Kennedy

DEPARTMENT OF RADIATION ONCOLOGY, UNIVERSITY OF PENNSYLVANIA SCHOOL OF MEDICINE,
PHILADELPHIA, PA 19104, USA

ABSTRACT. Certain protease inhibitors, called the anticarcinogenic protease inhibitors in this review, are capable of preventing carcinogenesis in a wide variety of *in vivo* and *in vitro* model systems. The anticarcinogenic protease inhibitors are extremely potent agents with the ability to prevent cancer, with some unique characteristics as anticarcinogenic agents. The anticarcinogenic protease inhibitors have the ability to irreversibly suppress the carcinogenic process. They do not have to be continuously present to suppress carcinogenesis. They can be effective when applied in both *in vivo* and *in vitro* carcinogenesis assay systems at long time periods after carcinogen exposure, and are effective as anticarcinogenic agents at extremely low molar concentrations. While several different types of protease inhibitors can prevent the carcinogenic process, the most potent of the anticarcinogenic protease inhibitors on a molar basis are those with the ability to inhibit chymotrypsin or chymotrypsin-like proteases. The soybean-derived protease inhibitor, Bowman-Birk inhibitor (BBI), is a potent chymotrypsin inhibitor that has been extensively studied for its ability to prevent carcinogenesis in many different model systems. Much of this review is focused on the characteristics of BBI as the anticarcinogenic protease inhibitor, as this is the protease inhibitor that has risen to the human trial stage as a human cancer chemopreventive agent. Part of this review hypothesizes that the Bowman-Birk family of protease inhibitors plays a role in plants similar to that of α_1-antichymotrypsin in people. Both BBI and α_1-antichymotrypsin are potent inhibitors of chymotrypsin and chymotrypsin-like enzymes, are highly anti-inflammatory, and are thought to play important roles in the defense of their respective organisms. It is believed that BBI will be shown to play a major role in the prevention and/or treatment of several different diseases, in addition to cancer. PHARMACOL. THER. 78(3):167–209, 1998. © 1998 Elsevier Science Inc.

KEYWORDS. Protease inhibitor, carcinogenesis, inflammation, Bowman-Birk inhibitor.

CONTENTS

ABBREVIATIONS. AM, α_2-macroglobulin; BBI, Bowman-Birk inhibitor; BBIC, Bowman-Birk inhibitor concentrate; CCK, cholecystokinin; CI, chymotrypsin inhibitor; DFP, diisopropyl fluorophosphate; DMBA, 7,12-dimethylbenz(a)anthracene; DMH, dimethylhydrazine; FDA, Food and Drug Administration; GI, gastrointestinal; IBD, inflammatory bowel disease; IME, intermediate marker endpoints; MBNA, N-nitrosomethylbenzylamine; PA, plasminogen activator; PBBI, purified Bowman-Birk inhibitor; PKC protein kinase C; PMA, phorbol-myristate-acetate; PMN, polymorphonuclear leukocytes; SBTI, soybean trypsin inhibitor; SRI, Southern Research Institute; $t_{1/2}$, serum half-life; TI, trypsin inhibitor; TPA, 12-O-tetradecanoyl-phorbol-13 acetate; UC, ulcerative colitis.

1. SUMMARY OF DATA SHOWING THE ANTICARCINOGENIC ACTIVITY OF PROTEASE INHIBITORS

Protease inhibitors are now well established as a class of cancer chemopreventive agents. While several different types of protease inhibitors have been shown to prevent the carcinogenic process, the most potent of the anticarcinogenic protease inhibitors on a molar basis are those with the ability to inhibit chymotrypsin or chymotrypsin-like proteases. There are several protease inhibitors that are part of the human diet that have the ability to inhibit chymotrypsin-like enzymes. One potent chymotrypsin inhibitor (CI), the soybean-derived protease inhibitor known as the Bowman-Birk inhibitor (BBI), has been studied extensively for its ability to prevent carcinogenesis in both in vivo and in vitro systems.

BBI has been studied both as purified BBI (PBBI) and as an extract of soybeans enriched in BBI called BBI concen-

trate (BBIC). PBBI and BBIC have comparable suppressive effects on the carcinogenic process in a variety of in vivo and in vitro systems. BBI appears to be a universal cancer preventive agent. PBBI/BBIC have been shown to suppress carcinogenesis: (1) in three different species (mice, rats, and hamsters); (2) in several organ systems/tissue types [colon, liver, lung, esophagus, cheek pouch (oral epithelium), and in cells of hematopoietic origin]; (3) in cells of both epithelial and connective tissue origin; (4) when given to animals by several different routes of administration (including the diet); (5) leading to different types of cancer (e.g., squamous cell carcinomas, adenocarcinomas, angiosarcomas, etc.); and (6) induced by a wide variety of chemical and physical carcinogens. BBI, as BBIC, recently has risen to the human trial stage. BBIC achieved Investigational New Drug status from the Food and Drug Administration (FDA) as of April, 1992, and studies to evaluate BBIC as an

anticarcinogenic agent in human populations have begun. Both BBI and BBIC prevent/suppress malignant transformation in vitro and carcinogenesis in vivo without toxicity.

The protease inhibitors discussed in this report are extremely potent agents having the ability to prevent cancer, with some unique characteristics as anticarcinogenic agents. The anticarcinogenic protease inhibitors have the ability to irreversibly suppress the carcinogenic process, they do not have to be continuously present to suppress carcinogenesis, they can be effective when applied in both in vivo and in vitro carcinogenesis assay systems at long time periods after carcinogen exposure, and they are effective as anticarcinogenic agents at extremely low molar concentrations. Much of this review is focused on the characteristics of BBI as the anticarcinogenic protease inhibitor, as this is the protease inhibitor that has risen to the human trial stage as a human cancer chemopreventive agent. As part of this review, it is hypothesized that the Bowman-Birk family of protease inhibitors plays a role in plants similar to that of α_1-antichymotrypsin in humans. Both BBI and α_1-antichymotrypsin are potent inhibitors of chymotrypsin and chymotrypsin-like enzymes, they are highly anti-inflammatory, and they are thought to play important defense roles in their respective organisms. It is believed that BBI will be shown to play a major role in the prevention and/or treatment of several different diseases in addition to cancer.

1.1. Epidemiologic Data Suggesting the Anticarcinogenic Activity of Protease Inhibitors

Cancer incidence and mortality rates vary greatly around the world, and dietary factors are thought to play a very important role in these variable rates, as has been reviewed elsewhere (e.g., Grobstein et al., 1982; Doll and Peto, 1981; Correa, 1981; Phillips, 1975). There are now many different epidemiologic studies that suggest that components of vegetables, particularly legumes (Correa, 1981), might play a beneficial role in lowering the incidence rates of many different types of cancer (some examples of such studies are given in the references cited above). Legumes are known to contain high levels of protease inhibitors (Birk, 1974, 1976, 1985; Kassell, 1970). In the diet, rice, maize, and beans, all of which contain high levels of protease inhibitors (Birk, 1974, 1976), are associated with a reduced incidence of colon, breast, and prostate cancers (Correa, 1981). The intake of breads and cereals, food sources that are also known to contain high levels of protease inhibitors, has been associated with a lowered incidence of oral and pharyngeal cancers (Winn et al., 1984). Other studies with high levels of protease inhibitors in the diet have also suggested a reduced risk of developing colorectal and breast cancers (Blondell, 1988). The role of vegetable-derived protease inhibitors as active cancer chemopreventive agents has been reviewed recently (e.g., Fontham and Correa, 1993).

There is evidence for a particularly strong relationship between the ingestion of soybeans and low incidence and mortality rates for breast, colon, and prostate cancers, as discussed elsewhere (e.g., Messina and Barnes, 1991; Messina et al., 1994). Although breast, colon, and prostate cancers are major public health problems in the Western world, cancer rates in these organs are significantly lower in most countries of the Pacific basin (e.g., Tominaga, 1985). When Orientals migrate to the United States, their offspring develop the common Western cancers at approximately the same rates as do Americans (Dunn, 1975; Haenszel and Kurihara, 1968; Buell, 1973). This observation has suggested that it is not a genetic difference in susceptibility to breast, colon, and prostate cancer development that results in the variation in cancer rates, but instead it is more likely due to variables such as dietary habits. It is known that the consumption of soybean products in countries such as Japan is very high (Sipos, 1990); it has been suggested that it is the large-scale consumption of soybean products in such countries that leads to their extremely low cancer mortality rates (Messina, 1995; Messina and Barnes, 1991; Messina et al., 1994). Soybeans are known to contain very high levels of protease inhibitors (Hwang et al., 1977; Rackis and Anderson, 1964). There are a number of potential anticarcinogenic agents in soybeans, which include saponins, isoflavones, and other agents, as reviewed by Messina and Barnes (1991). Although two other agents in soybean [specifically, phytic acid (inositol hexahisphosphate) and the sterol β-sitosterol] have been shown to suppress carcinogenesis in animals, BBI is far more effective at suppressing the yields of cancers in animals than the other known anticarcinogenic agents in soybeans, as described in detail elsewhere (Kennedy, 1995a). Thus, it has been hypothesized that it is the high level of dietary protease inhibitors that has led to low cancer mortality rates in populations ingesting large amounts of soybean products (Kennedy, 1993a; Kennedy et al., 1993b). The epidemiologic studies establish the plausibility of this hypothesis, but since there are always alternative explanations for variations in cancer incidence and mortality data, epidemiologic studies cannot prove this hypothesis.

1.2. Experimental Data Involving Carcinogenesis Model Systems

While there are a number of explanations for the epidemiologic data on diet and cancer frequency, conclusive results concerning the role of dietary additives can be obtained from well-controlled animal studies. There are laboratory data demonstrating unequivocally a strong cancer preventive role for dietary protease inhibitors in several different animal model carcinogenesis systems (e.g., Troll, 1976; Troll et al., 1970, 1979a,b, 1980; Becker, 1981; Yamauchi et al., 1987; Ohkoshi and Fujii, 1983; Matsushima et al., 1976; Slaga et al., 1980; Corasanti et al., 1982; Yamamoto et al., 1974; Yamamura et al., 1978; Nomura et al., 1980; Berenblum et al., 1974; Kennedy and Billings, 1987; Umezawa, 1972; Rossman and Troll, 1980; Hozumi et al., 1972; Weed et al., 1985; Messadi et al., 1986; Witschi and Kennedy, 1989; St. Clair et al., 1990a; Billings et al., 1990c; von Hofe et al., 1991; Kennedy et al., 1993a,b; Kennedy, A. R. et al.,

1996); these in vivo data are summarized in Table 1. The dietary protease inhibitor that has been studied most extensively as a cancer preventive agent is the soybean-derived protease inhibitor BBI; many of the studies listed in Table 1 have been performed with BBI as the active anticarcinogenic agent.

Both the plant and animal kingdoms contain a variety of protease inhibitors. They are found in many common foods, including legumes, cereals, oilseeds, nuts, fruits, vegetables, eggs, potatoes, and other dairy and animal products. Many vegetables contain protease inhibitors that could be useful as cancer chemopreventive agents, as has been reviewed (Birk, 1974, 1976, 1985, 1993; Liener and Kakade, 1980; Rackis et al., 1986). Soybeans are unusually rich in protease inhibitor activity; protease inhibitors comprise at least 6% of the total soybean protein (Hwang et al., 1977; Rackis and Anderson, 1964). Several protease inhibitors are present in soybeans. The best characterized of these inhibitors are soybean trypsin inhibitor (SBTI) (Kunitz, 1947) and BBI (Bowman, 1946, 1993; Birk, 1961, 1974, 1976, 1985). SBTI has a molecular weight of about 21,000 and has primarily trypsin inhibitory (TI) activity; BBI has a molecular weight of about 8000 and inhibits chymotrypsin and trypsin (Birk, 1974, 1976). The other soybean protease inhibitors have not been as fully characterized as BBI and SBTI, but are known to inhibit trypsin (Hwang et al., 1977). The Bowman-Birk family of protease inhibitors exists in many different kinds of plants, but is particularly prominent in legumes, many of which are a normal part of the human diet. The anticarcinogenic activity of the soybean-derived BBI was initially discovered when it was shown to prevent radiation-induced transformation in vitro (Yavelow et al., 1983). Other members of the Bowman-Birk family of protease inhibitors (e.g., the chickpea inhibitor) are also capable of preventing the malignant transformation of cells; the Bowman-Birk family of protease inhibitors presumably inhibits carcinogenesis due to its ability to inhibit chymotrypsin (Yavelow et al., 1985; Kennedy, 1985a). Certain other protease inhibitors have the ability to inhibit chymotrypsin. For example, the CI from potatoes inhibits chymotrypsin (Birk, 1976; Ryan and Kassell, 1970; Smirnoff et al., 1979); unlike the Bowman-Birk type of protease inhibitors, this protease inhibitor does not have a distinct TI region. Fruits are also known to contain protease inhibitors that inhibit chymotrypsin; for example, bananas are known to have a particularly high CI content (Rackis et al., 1986).

A number of different protease inhibitors were shown to prevent the malignant transformation of cells in vitro; these in vitro studies demonstrating the anticarcinogenic activity of certain protease inhibitors have been reviewed recently (Kennedy, 1993b) and are summarized in Table 2. For the purpose of this review, anticarcinogenic protease inhibitor will be defined as a protease inhibitor that has been shown to prevent cancer in animals or carcinogen-induced transformation in vitro in a statistically significant fashion. [Other types of "anticarcinogenic" activity have been reported for protease inhibitors as well, as described elsewhere (e.g., Troll and Kennedy, 1993)]. Many of the protease inhibitors

shown to inhibit malignant transformation in vitro were considered as potential agents to develop as human cancer preventive agents. As described elsewhere (Kennedy, 1993a), it is believed that the important properties of an ideal protease inhibitor to be used as a cancer chemopreventive agent include the ability to inhibit transformation in vitro; to have chemical stability, water solubility, and the abilities to inhibit the enzyme chymotrypsin; to be internalized by cells (for a discussion of this, see Billings et al., 1989, 1990a, 1991a); and to lack the ability to inhibit the blood clotting process. BBI has all of these properties, which are considered important. Thus, for a number of different reasons, BBI was chosen as the most hopeful of the dietary protease inhibitors for development into a human cancer preventive agent (Kennedy, 1993a). Recently, human trials have begun in which BBI, in the form of BBIC, is being evaluated for its ability to prevent cancer. Studies with BBI/BBIC are described below.

2. THE PROPERTIES OF ANTICARCINOGENIC PROTEASE INHIBITORS, WITH EMPHASIS ON THE SOYBEAN-DERIVED BOWMAN-BIRK INHIBITOR

2.1. Purified Bowman-Birk Inhibitor and Bowman-Birk Inhibitor Concentrate as Cancer Preventive Agents

The work of the Kennedy laboratory focused initially on the properties of several different protease inhibitors as agents capable of preventing the malignant transformation of cells in in vitro systems. It soon became clear that agents that had the ability to suppress malignant transformation in vitro were acting in a similar fashion, suggesting a common mechanism of action. As research began to focus on the development of an agent for human cancer prevention trials, more and more of the research investigations utilized BBI as the anticarcinogenic protease inhibitor. Thus, as BBI is the protease inhibitor that has risen to the human trial stage, far more is known about BBI than any of the other known anticarcinogenic protease inhibitors. The characteristics of BBI as a protease inhibitor that is capable of preventing cancer will be described in detail here.

The discovery of BBI as a dietary protease inhibitor has been described previously (Bowman, 1993; Birk, 1993). BBI is a relatively well-characterized protease inhibitor, whose complete structure has been determined by Odani and Ikenaka (1973); the structure of BBI is shown in Fig. 1A. A computer-simulated three-dimensional structure of BBI in solution is shown in Fig. 1B. The structure of BBI in solution was determined by two-dimensional ^{1}H nuclear magnetic resonance spectroscopy and dynamical-simulated annealing, as described by Werner and Wemmer (1992).

2.1.1. Rationale for production of Bowman-Birk inhibitor concentrate. BBI proved to be so effective in its ability to suppress malignant transformation in vitro that it was

TABLE 1. Protease Inhibitor Suppression of Carcinogenesis *in Vivo*

Carcinogen	Protease inhibitor	Animal model system	Reference
DMBA (promotor = croton oil or phorbol ester)	TLCK[1] TPCK[2] TAME[3]	Mouse skin tumorigenesis	Troll et al., 1970
DMBA (promotor = croton oil)	Leupeptin	Mouse skin tumorigenesis	Hozumi et al., 1972
Azoxymethane	Leupeptin	Rat colon carcinogenesis	Yamamoto et al., 1974
Radiation	AM	Mouse lymphatic leukemia	Berenblum et al., 1974
Chemical carcinogenesis	Leupeptin	Rat colon, esophagus, and mammary gland carcinogenesis; Mouse leukemia, skin carcinogenesis	Matsushima et al., 1976
DMBA	N.N-dimethylamino (p-p'-guanidinobenzoyloxy) benzilcarbonyloxyglycolate	Rat mammary tumorigenesis	Yamamura et al., 1978
4-Nitroquinoline oxide and PMA	Soybean diet	Mouse skin tumorigenesis	Troll et al., 1979a,b
Urethane	Antipain	Mouse lung tumorigenesis	Nomura et al., 1980
X-irradiation	Soybean diet	Rat mammary carcinogenesis	Troll et al., 1980
DMBA	TPCK[2]	Mouse skin tumorigenesis	Slaga et al., 1980
(Spontaneous)	Edi Pro A (isolated soy protein)	Mouse liver tumorigenesis	Becker, 1981
Dimethylhydrazine	Σ=Aminocaproic acid	Mouse colon tumorigenesis	Corasanti et al., 1982
3-Methylcholanthrene	[N-N-dimethyl carbamoylmethyl 4-(4-guanidinobenzoyloxy)-phenylacetate] methanesulfate (FOY-305)	Mouse skin carcinogenesis	Ohkoshi and Fujii, 1983
Dimethylhydrazine	Bowman-Birk	Mouse colon tumorigenesis	Weed et al., 1985; St. Clair et al., 1990a; Billings et al., 1990c
DMBA	Bowman-Birk	Hamster oral carcinogenesis	Messadi et al., 1986; Kennedy et al., 1993a
	FOY-305	Rat liver carcinogenesis	Yamauchi et al., 1987
3-Methylcholanthrene	Bowman-Birk	Mouse lung tumorigenesis	Witschi and Kennedy, 1989
Dimethylhydrazine	Bowman-Birk	Mouse liver tumorigenesis	St. Clair et al., 1990a
MBNA	Bowman-Birk	Rat esophageal carcinogenesis	von Hofe et al., 1991
(Spontaneous) genetic susceptibility	Bowman-Birk	Mouse-Min	Kennedy et al., 1996

[1]Tosyl-lysine chloromethyl ketone.
[2]Tosyl-phenylalanine chloromethyl ketone.
[3]Tosyl-arginine methyl ester.

deemed necessary to determine its ability to suppress carcinogenesis in animal model systems. As a soybean extract containing the inhibitor would be of greater applicability in future animal and human cancer chemopreventive studies, various soybean extracts containing BBI were evaluated as potential anticarcinogenic preparations. Unfortunately, finding an effective extract proved to be a difficult task, for unknown reasons. Many of the extracts that were produced were not effective as anticarcinogenic agents in *in vitro* transformation assays, and some even enhanced transformation [e.g., soybean flour, as discussed in Yavelow et al. (1985)]. There are, in fact, some compounds in soybeans, such as the soybean lectin, that might be expected to work like a promoting agent for carcinogenesis. It was also clear that compounds are present in soybeans that are able to mask the ability of BBI to serve as an anticarcinogenic agent and that are removed by various purification procedures, such as those described by Birk (Kassell, 1970) and

Hwang et al. (1977). Pure BBI is an effective anticarcinogenic agent when produced by either the Birk (Kassell, 1970) or the Hwang et al. (1977) procedures; however, none of the "intermediate" extracts produced by either of those published procedures have anticarcinogenic activity [as measured by the ability to suppress radiation-induced transformation *in vitro* (Kennedy, 1993a)]. An anticarcinogenic extract of soybeans was produced by acetone pretreatment of the starting material followed by the Birk purification procedure, as described in detail elsewhere (Yavelow et al., 1985); this extract is known as BBIC. BBIC has the same ability as pure BBI to suppress malignant transformation *in vitro*; the anticarcinogenic activity of BBIC is thought to be due solely to the presence of BBI (Yavelow et al., 1985). BBIC contains five separate soybean protease inhibitors (including BBI, but not including SBTI), and all of which are members of the Bowman-Birk family of inhibitors. Only BBI itself, however, in BBIC has the ability to

TABLE 2. Protease Inhibitors with the Ability to Suppress Carcinogen-Induced Malignant Transformation *in Vitro*

Protease inhibitor	Carcinogen	First reference utilizing each protease inhibitor
Antipain	Radiation	Kennedy and Little, 1978
Leupeptin		Kennedy and Little, 1978
Antipain	Radiation	Borek et al., 1979
Antipain	3-Methylcholanthrene	Kuroki and Drevon, 1979
Chymostatin		
Elastatinol		
Leupeptin		
Pepstatin		
Antipain	N-methyl-N'-nitro-N-nitroso-guanidine	DiPaolo et al., 1980
Antipain	Radiation/17-βEstradiol	Kennedy and Weichselbaum, 1981
Leupeptin		
BBI	Radiation	Yavelow et al., 1983, 1985
Chymostatin	Radiation	Kennedy, 1984, 1985a
TPCK[1]	Radiation	Kennedy, 1985a
BBI (fragment containing chymotrypsin inhibitory site)	Radiation	Yavelow et al., 1985
BBIC	Radiation	Yavelow et al., 1985
	Radiation, benzo(a)pyrene, β-propiolactone (with and without enhancement by pyrene)	Baturay and Kennedy, 1986
Chickpea inhibitor	Radiation	Yavelow et al., 1985
Chymotrypsin inhibitor I from potatoes	Radiation	Billings et al., 1987
Carboxypeptidase inhibitor I and inhibitor II from potatoes	Radiation	Billings et al., 1989
Aprotinin and N-acetyl-L-tyrosine ethyl ester	Radiation	Billings et al., 1989
BBI-Polylysine conjugate	Radiation	Persiani et al., 1991
BBI	3-Methylcholanthrene	St. Clair, 1991
Edi Pro A (soybean extract prepared by Ralston Purina)	Radiation	Kennedy, 1993b
E-amino-n-caproic acid	Radiation	Kennedy, 1993b
Succinylated BBI-BBI lacking TI site	Radiation	Kennedy, 1993b
Spermine-BBI conjugate	Radiation	Ekrami et al., 1993
BBI-Polyester conjugate	Radiation	Larionova et al., 1994a
BBI-Palmitic acid derivatized conjugate	3-Methylcholanthrene	Ekrami et al., 1995b

[1]Tosyl-phenylalanine chloromethyl ketone.

inhibit chymotrypsin. The other trypsin inhibitors in BBIC have been only partially characterized (Hwang et al., 1977). It has been observed that BBIC has essentially the same ability as PBBI to suppress the carcinogenic process in both *in vitro* (e.g., Yavelow et al., 1985; Baturay and Kennedy, 1986) and *in vivo* (e.g., St. Clair et al., 1990a; Kennedy et al., 1993a) systems.

Perhaps the most important reason for the production of a BBI-containing preparation rather than use of whole soybeans as an anticarcinogenic agent is that much of the TI activity of the soybean extract can be removed. As described in Sections 2.4 and 5.2.1, it is known that it is the TI activity in soybeans that is involved in triggering a pancreatic feedback response in rats, while the chymotrypsin inhibitory activity of BBI/soybeans is not involved in this potentially toxic effect. Normally, there is far more trypsin inhibitor activity in raw soybeans than chymotrypsin inhibitory activity. As discussed in Section 2.1.2, the TI activity has been greatly reduced, such that it is only a fraction of the protease inhibitory activity present in BBIC.

2.1.2. Characteristics of purified Bowman-Birk inhibitor and Bowman-Birk inhibitor concentrate.

The methods of production of BBIC from acetone-defatted soybean flour are described in detail elsewhere (Yavelow et al., 1985), as are its characteristics (Kennedy et al., 1993b). A purified form of BBI, termed PBBI, is prepared from BBIC by DEAE-cellulose ion-exchange chromatography; it has been estimated that these methods of production of PBBI result in a product purified to near homogeneity (one major protein band is present on sodium dodecyl sulfate-polyacrylamide gels), which is greater than 95% pure (St. Clair et al., 1990a). BBIC has been well characterized and described in detail (Kennedy et al., 1993b). BBIC contains proteins and carbohydrates, with essentially no fat. In BBIC, the active anticarcinogenic activity has been shown to be CI activity, which is present in soybeans only in BBI (Birk, 1974, 1976, 1985; Kassell, 1970; Hwang et al., 1977). The doses of BBIC are measured in CI units, as described in detail elsewhere (Kennedy et al., 1993b); BBIC contains 100 mg/g CI activity, and, at most, 40 mg/g TI activity. The CI activity in
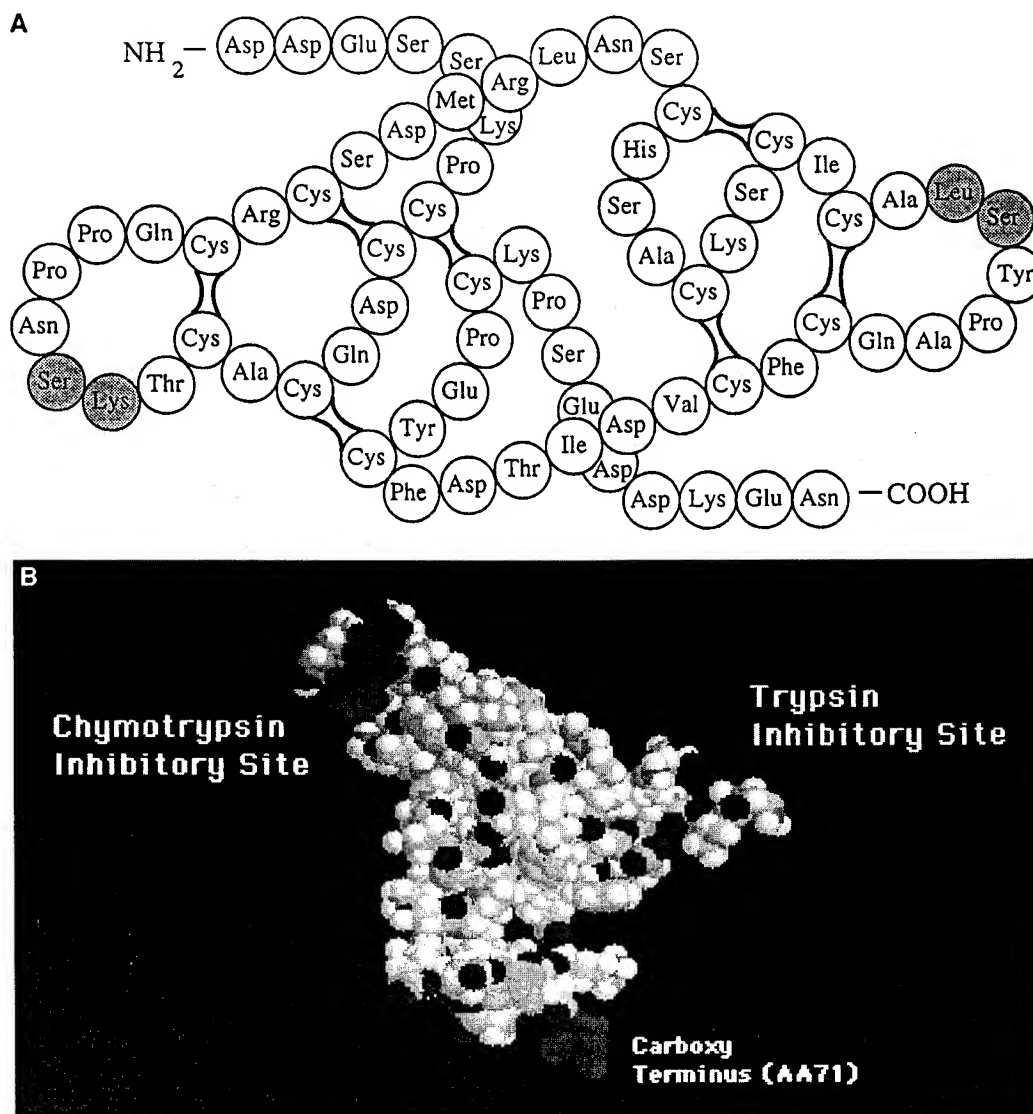
FIGURE 1. (A) Structure of BBI, as determined by Odani and Ikenaka (1973). In the drawing, the chymotrypsin inhibitory site appears on the right side (Leu-Ser; darkened circles) and the trypsin inhibitory site appears on the left side (Lys-Ser, darkened circles). Reproduced from Odani and Ikenaka (1973), with permission of the authors and the copyright holder, Japanese Biochemical Society, Tokyo. (B) Three-dimensional computer-simulated structure of BBI in solution. The BBI is a 71 amino acid (AA) protein. The end of BBI is AA71, the carboxy terminus of the protein is shown in purple. The beginning of BBI, AA1, is shown in yellow. The chymotrypsin inhibitory site, AA43-44, is shown in green. The GI site, AA16-17, is shown in blue. Reproduced from Kennedy (1994), with permission of the copyright holder, American Chemical Society, Washington, DC.

BBIC is known to be stable over at least a 2-year period, as is the ability of BBIC to inhibit transformation *in vitro* (Kennedy *et al.*, 1993b). BBIC has achieved Investigational New Drug status from the FDA, and trials to evaluate BBIC as an anticarcinogenic agent in human populations began in 1992.

2.2. Review of the Data on the Anticarcinogenic Activity of Bowman-Birk Inhibitor

2.2.1. Review of the *in vivo* anticarcinogenic activity of Bowman-Birk inhibitor. The anticarcinogenic activities of both PBBI and BBIC have been studied extensively; PBBI works as well as BBIC as an anticarcinogenic agent

over a range of doses in both *in vitro* transformation systems and in *in vivo* carcinogenesis assay systems (e.g., Yavelow *et al.*, 1985; Kennedy *et al.*, 1993a; St. Clair *et al.*, 1990a). As discussed in Section 1.2, BBI/BBIC prevent or suppress radiation- and chemical carcinogen-induced malignant transformation *in vitro* (Yavelow *et al.*, 1983, 1985; Kennedy, 1985a; Baturay and Kennedy, 1986; St. Clair, 1991) and carcinogenesis in animals in several different model systems. It has been observed that BBI and/or BBIC suppress carcinogenesis: (1) in three different species (mice, rats, hamsters); (2) in several different organ systems/tissue types [colon, lung, liver, esophagus, cheek pouch (oral epithelium), and in cells of hematopoietic origin]; (3) in different

cell types [epithelial cells (in the colon, liver, lung, esophagus and cheek pouch)], as well as connective tissue cells (fibroblasts *in vitro* and those in the liver that give rise to angiosarcomas); (4) when given to animals by several different routes of administration [including injection (i.p. or i.v.), direct application, and through the diet]; and (5) leading to different types of cancers [e.g., squamous cell carcinomas (oral epithelium), adenocarcinomas (colon), angiosarcomas (liver), etc.]. The anticarcinogenic activities of PBBI/BBIC have been studied in several different *in vivo* carcinogenesis model systems (Weed *et al.*, 1985; Messadi *et al.*, 1986; Witschi and Kennedy, 1989; St. Clair *et al.*, 1990a; Billings *et al.*, 1990c; von Hofe *et al.*, 1991; Evans *et al.*, 1992; Kennedy *et al.*, 1993a; Kennedy *et al.*, 1996) and in several different *in vitro* systems (Yavelow *et al.*, 1983, 1985; Kennedy *et al.*, 1984b; Kennedy, 1982, 1984, 1985a, 1988, 1990, 1993b; Baturay and Kennedy, 1986; Umans *et al.*, 1985; Billings, 1993; Billings *et al.*, 1987, 1988, 1989, 1991a,b; Billings and Habres, 1992; Caggana and Kennedy, 1989; Carew and Kennedy, 1990; Chang *et al.*, 1985, 1990; Chang and Kennedy, 1988, 1993; Flick and Kennedy, 1991; Persiani *et al.*, 1991; Su *et al.*, 1991; St. Clair *et al.*, 1991; Ekrami *et al.*, 1993, 1995b; Li *et al.*, 1992; Umans *et al.*, 1984, 1985). In addition to these studies, a number of studies have been performed to relate the *in vitro* findings on PBBI/BBIC to *in vivo* effects (St. Clair *et al.*, 1990b; Oreffo *et al.*, 1991; Billings *et al.*, 1992; Maki and Kennedy, 1992; Ekrami *et al.*, 1993; St. Clair and St. Clair, 1991; Kennedy and Little, 1978, 1981a; Kennedy *et al.*, 1984b, 1993b). There are several reviews on studies utilizing PBBI/BBIC (Kennedy, 1984, 1988, 1990, 1993a,b,c,d,e, 1994, 1995a,b; Kennedy *et al.*, 1993b; Kennedy and Billings, 1987).

The specific animal model carcinogenesis systems in which BBI and/or BBIC have been shown to have a suppressive effect on carcinogenesis include (1) dimethylhydrazine (DMH)-induced colon (Weed *et al.*, 1985; St. Clair *et al.*, 1990a; Billings *et al.*, 1990c) and liver (St. Clair *et al.*, 1990a) carcinogenesis in mice, (2) 7,12-dimethylbenz(a)anthracene (DMBA)-induced oral carcinogenesis in hamsters (Messadi *et al.*, 1986; Kennedy *et al.*, 1993a), (3) 3-methylcholanthrene-induced lung carcinogenesis in mice (Witschi and Kennedy, 1989), (4) methylbenzylnitrosamine-induced esophageal carcinogenesis in rats (von Hofe *et al.*, 1991), (5) radiation-induced thymic lymphosarcoma in mice (Evans *et al.*, 1992), and (6) spontaneous colon carcinogenesis in mice that are genetically susceptible to the induction of intestinal carcinogenesis (Kennedy, A. R. *et al.*, 1996). For radiation-induced lymphosarcoma, BBIC was shown to prevent the extension and metastasis of tumors (Evans *et al.*, 1992). Several of these *in vivo* studies have been reviewed elsewhere (Kennedy and Billings, 1987; Kennedy, 1993a) and are summarized in Section 2.2.1.1.

It is believed that the great strength of BBI as a cancer preventive agent lies in its ability to reverse the "initiated" state of cells after initiation has occurred; few other anticarcinogenic agents are capable of "inactivating" initiated cells (for a review of this area of research, see Kennedy,

1994). It is expected that some other potential cancer preventive agents will be able to prevent the initiation of cells, but the ability to reverse initiation is essentially a unique characteristic of BBI as a cancer preventive agent. The ability to reverse initiation was observed first in *in vitro* studies (Kennedy, 1985a); similar results suggesting the reversal of the initiated state have been observed in *in vivo* carcinogenesis studies (Kennedy *et al.*, 1993a). In animal carcinogenesis studies, BBI leads to a reduction in the yields of both tumors and premalignant lesions in a variety of systems (e.g., Kennedy, 1993a; Kennedy *et al.*, 1993a; St. Clair *et al.*, 1990a; von Hofe *et al.*, 1991). Both PBBI and BBIC are effective as anticarcinogenic agents, even when given at very long time periods after carcinogen exposure, in both *in vivo* and *in vitro* studies (Kennedy *et al.*, 1993a; Kennedy, 1985a). In animal studies on both oral (Kennedy *et al.*, 1993a) and colon (unpublished data) carcinogenesis, BBI/BBIC treatment can begin 3 months after carcinogen exposure in a 6-month assay period and still have a suppressive effect on carcinogenesis. In systems such as these, it is known that premalignant lesions can be observed at 3 months, as determined from serial sacrifice experiments. As the yields of both tumors and premalignant lesions are markedly reduced in BBI-treated animals, these results suggest that BBI treatment has destroyed the premalignant lesions. It is now known that BBI/BBIC treatment can result in toxicity for premalignant and some malignant cells *in vitro*, as described in Section 2.2.2.2.

2.2.1.1. *Details of studies of Bowman-Birk inhibitor/Bowman-Birk inhibitor concentrate in animal carcinogenesis assay systems.* In the colon, BBI completely prevented carcinogenesis induced by a low carcinogen dose (Weed *et al.*, 1985; St. Clair *et al.*, 1990a) and suppressed carcinogenesis induced by a high carcinogen dose (Billings *et al.*, 1990c). It was observed that PBBI and BBIC had the same suppressive effects on liver and colon carcinogenesis induced by DMH (St. Clair *et al.*, 1990a). BBI affected adenomatous tumors of the mouse colon, which are histopathologically similar to those that occur in the most common form of human colon cancer. BBI was also shown to reduce the numbers of tumors in both the small intestine and colon in Min mice (Kennedy, A. R. *et al.*, 1996), a strain of mice with an autosomally dominantly inherited predisposition to multiple intestinal neoplasms. Min mice are known to carry a mutation that is similar to the mutation occurring in familial adenomatous polyposis patients and that is thought to lead to their significantly elevated incidence and mortality rates from intestinal cancer, as reviewed elsewhere (Kennedy, A. R. *et al.*, 1996). BBI reduced both the total number of intestinal tumors occurring in Min mice and the fraction of Min mice having colon tumors. The BBI suppression of intestinal tumorigenesis in Min mice was not accompanied by an effect on animal growth. In the liver, BBI suppressed the yield of premalignant lesions, such as hyperplasia, as well as the DMH-induced elevation in the incidence of angiosarcomas in mice (St. Clair *et al.*, 1990a). For both angiosarcomas

and hyperplasia in the liver, PBBI worked as well as BBIC as a cancer preventive agent. In both the colon and liver carcinogenesis studies, BBI was administered to the animals via the diet.

Both PBBI and BBIC have been shown to prevent oral carcinogenesis in a statistically significant fashion (Messadi et al., 1986; Kennedy et al., 1993a); in these studies, BBI was topically applied to the hamster cheek pouch. As part of these studies, it was observed that PBBI and BBIC had comparable abilities to prevent DMBA-induced oral carcinogenesis in hamsters and that protease inhibitors could prevent cancer in this system even when administered to animals as long as 3 months following carcinogen exposure (Kennedy et al., 1993a). In addition, it was observed that protease inhibitors had an irreversible suppressive effect on the carcinogenic process in animals, since they could be removed from animals months before the end of the assay period and still have a significant suppressive effect on DMBA-induced oral carcinogenesis (Kennedy et al., 1993a). Data supporting these concepts are shown in histogram form in Fig. 2. BBI was also shown to inhibit esophageal carcinogenesis induced by N-nitrosomethylbenzylamine (MBNA); in this study, BBI was administered to the animals as a lozenge formulation, as described elsewhere (von Hofe et al., 1991). A suppressive effect of BBI on both premalignant lesions and tumors of the esophagus was observed in MBNA-treated rats, without an effect on animal growth. In lung carcinogenesis studies, BBI has been given primarily as i.p. injections. BBI has been shown to reduce the percentage of animals bearing lung tumors, as well as the number of lung tumors per animal (Witschi and Kennedy, 1989). In one experiment, the suppression of tumorigenesis was compared when BBI was given i.p. vs. p.o. The results suggested that the same amount of BBI given p.o. was about one-half as effective as observed for the i.p. route of administration. BBI has shown to result in a reduction in premalignant lesions, as well as tumors, in many different organs.

2.2.1.2. *Summary of Bowman-Birk inhibitor/Bowman-Birk inhibitor concentrate in animal carcinogenesis assays.* For all studies involving BBIC, parallel studies utilizing PBBI showed essentially the same suppressive effect on animal carcinogenesis, allowing the conclusion that BBIC owed its anticarcinogenic activity to BBI (e.g., St. Clair et al., 1990a, Kennedy et al., 1993a; Witschi and Kennedy, 1989). For both the studies utilizing BBIC and PBBI, the degree of suppression of carcinogenesis was related to the dose of the carcinogenic agent used. If the dose of the carcinogenic agent was high, such that more than one-half of the exposed animals would be developing cancer, the incidence of animals developing tumors in the treatment group receiving PBBI or BBIC in addition to carcinogen treatment was approximately one-half that occurring in the animals receiving only the carcinogen treatment. If the dose of the carcinogenic agent was such that less than one-half of the exposed animals developed tumors, PBBI or BBIC had a greater sup-

pressive effect on carcinogenesis. In some of these experiments, it was observed that PBBI and BBIC completely prevented carcinogenesis (i.e., suppressed the carcinogenic process by 100%), as reviewed elsewhere (Kennedy, 1993a).

2.2.2. Review of the *in vitro* anticarcinogenic activity of Bowman-Birk inhibitor and other protease inhibitors.
2.2.2.1. *Prevention of transformation in vitro.* Much of the information about the anticarcinogenic activity of protease inhibitors comes from studies on the ability of these agents to suppress carcinogen-induced transformation *in vitro*. The anticarcinogenic protease inhibitors have the ability to completely suppress/prevent morphologic transformation in rodent cell systems, as illustrated in Fig. 3, as well as "transformation," as measured by anchorage-independent growth, in human diploid cell systems (Kennedy, 1993b,f). Different systems for measurement of *in vitro* transformation are described elsewhere (Kennedy, 1993b,f).

The anticarcinogenic protease inhibitors have the capability of completely abolishing carcinogen-induced transformation *in vitro* or promoter-enhanced transformation *in vitro*, as recently reviewed (Kennedy, 1993b). The highly significant suppressive effects of an anticarcinogenic protease inhibitor an radiation-induced transformation *in vitro* are shown in Fig. 3. The ability to prevent/suppress radiation transformation *in vitro* occurs in the absence of an effect on cell growth (Chang et al., 1985, 1990; Chang and Kennedy, 1988, 1993). It has been observed that several different types of protease inhibitors suppress carcinogen-induced malignant transformation *in vitro* (reviewed by Kennedy, 1993b), even when they are added to cells long after the radiation exposure (e.g., Kennedy, 1985a; reviewed by Kennedy, 1993b). Inhibitors of a specific protease, chymotrypsin, are the most effective for the suppression of malignant transformation (Kennedy, 1985a). BBI is a potent CI. Treatment of cells with low concentrations of CIs in the medium (e.g., at nanomolar concentrations) for short periods of time will suppress carcinogen-induced transformation; effective concentrations of protease inhibitors that do not inhibit chymotrypsin are many orders of magnitude higher than this (Kennedy, 1985a). It has been shown clearly that BBI and other protease inhibitors irreversibly inhibit carcinogen-induced transformation *in vitro* in a variety of cell systems; the anticarcinogenic protease inhibitors can suppress radiation transformation when applied to irradiated cultures as long as 2 weeks after the radiation exposure (e.g., Kennedy, 1985a). Protease inhibitors must be applied to proliferating cells to have a suppressive effect on transformation *in vitro*, and can be applied for as short a time period as 1 day to result in a suppression of transformation. The 1-day time period for protease inhibitor treatment has been shown to be effective when given to cultures either around the time of the radiation exposure or at 5 days post-irradiation (Kennedy and Little, 1978, 1981a; Kennedy, 1982; Yavelow et al., 1983, 1985). The ability of the anticarcinogenic protease inhibitors to suppress radiation transformation when applied to cultures for 1 day at
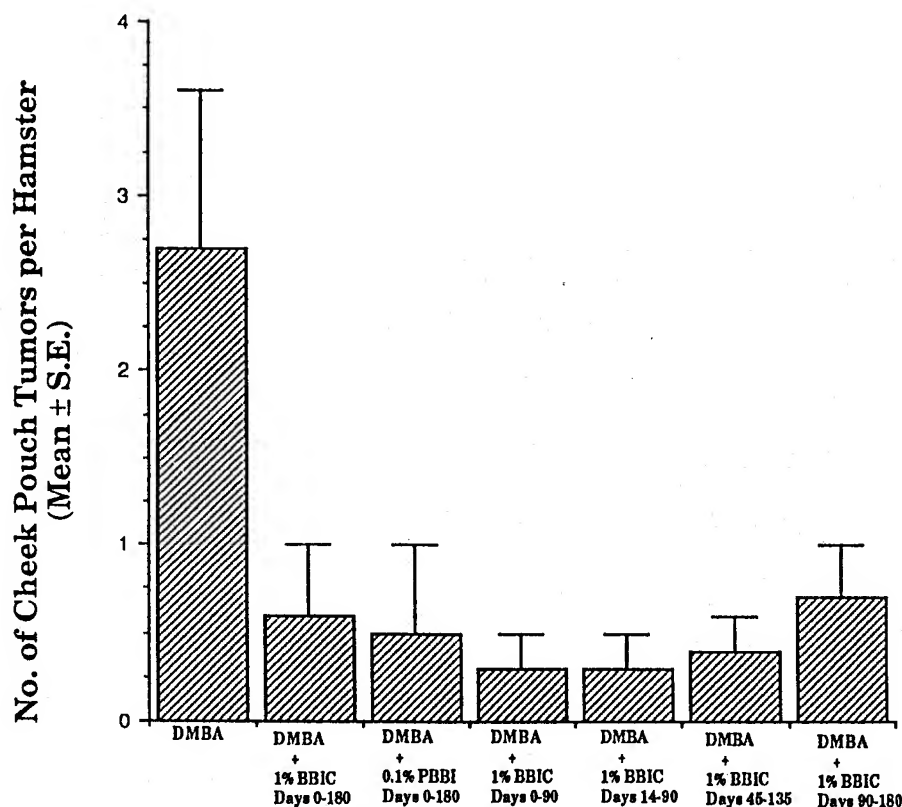
FIGURE 2. Suppressive effects of BBIC and PBBI on oral carcinogenesis induced in hamsters by DMBA. Note that PBBI and BBIC have essentially comparable suppressive effects on DMBA-induced oral carcinogenesis in hamsters, and that BBIC treatment can be given at a variety of times after carcinogen exposure in this system and still have a significant suppressive effect on the carcinogenic process. Of particular note are the treatment groups involving BBIC treatment from days 0 (or 14) to 90 and from days 90 to 180. When administered to animals for only the first 3 months of the 6-month experimental assay period, the suppressive effects of BBIC indicate that BBIC has an irreversible suppressive effect on the carcinogenic process. The fact that BBIC has a significant suppressive effect on the development of tumors when administered to animals for only the last 3 months of the carcinogenesis assay period indicates that protease inhibitors can suppress carcinogenesis in vivo, even when given at very long periods after carcinogen exposure. Data and details of these studies are given in Kennedy et al., 1993a. Reproduced from Kennedy (1994), with permission of the copyright holder, American Association for Cancer Research, Inc., Philadelphia.

day 5 post-irradiation is assumed to represent the reversal of the initiated state (Kennedy, 1982).

Much of the early work on the protease inhibitor suppression of radiation transformation utilized antipain as the anticarcinogenic protease inhibitor. Later work has demonstrated that BBI and other anticarcinogenic protease inhibitors have the same effects as antipain on radiation transformation in vitro (e.g., Yavelow et al., 1985); it is believed that the protease inhibitors with the ability to suppress transformation in vitro are working in a similar fashion and have a common mechanism of action. The major difference between protease inhibitors is the lowest molar concentration at which they have comparable effects. It is believed that in the rodent transformation systems, the anticarcinogenic protease inhibitors are capable of reversing the initiation of carcinogen-treated cells and affecting all stages of transformation in vitro, up to the formation of malignant cells. In in vitro transformation studies and other in vitro studies in which endpoints related to carcinogenesis were

studied, PBBI and BBIC had essentially comparable effects, and conclusions were drawn relating the anticarcinogenic effect of BBIC to BBI, as has been reviewed elsewhere (Kennedy, 1993b).

To discuss the mechanisms by which the anticarcinogenic protease inhibitors are modifying transformation in vitro, a summary of current thoughts on the mechanism(s) thought to be involved in the induction of malignant transformation by carcinogenic agents in the rodent transformation assay systems is necessary. From experiments performed with different initial and reseeded cell densities, it was concluded that the first event in carcinogen-induced malignant transformation is a high-frequency event occurring in many cells treated with low doses of either chemical or physical carcinogens (Kennedy et al., 1980, 1984a; Kennedy and Little, 1980, 1981b, 1984; Kennedy, 1984, 1985b, 1989, 1996b). It is believed that the hypothesized high-frequency initiating event is likely to be caused by a change in gene expression, as it is known that such changes
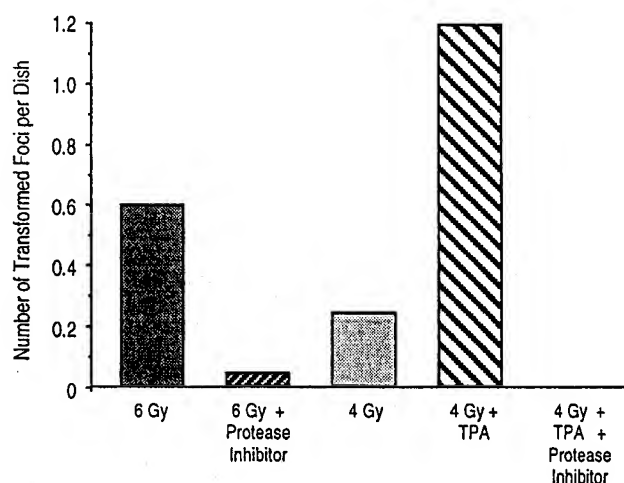
FIGURE 3. Effects of protease inhibitors on radiation-induced transformation *in vitro* in C3H10T1/2 cells. Protease inhibitors are capable of suppressing the TPA enhancement of radiation transformation, as well as the carcinogenic process brought about by a high dose of a complete carcinogen such as X-irradiation, as discussed in Kennedy (1984). Data from Kennedy and Little (1981a).

in gene expression can be caused by carcinogens (Fahmy and Fahmy, 1980) and can occur in a high proportion (≈80%) of carcinogen-treated cells (see, for example, Scott and Maercklein, 1985). These changes in gene expression can be thought of as acting like a switch (Kennedy, 1989, 1991), and are well known in radiation biology; such changes have been demonstrated even in irradiated human cells (Rosen and Klein, 1983). [Other similar inherited epigenetic changes are discussed elsewhere (Kennedy, 1985b, 1989)]. It is assumed that a major consequence of the (presumed) carcinogen-induced change in gene expression is that it confers on cells an altered probability that a subsequent event, malignant transformation (i.e., an event that leads directly to the malignant state), will occur (reviewed in Kennedy, 1985b, 1984). Previous work performed in the Kennedy laboratory has suggested that the anticarcinogenic protease inhibitors are able to stop what appears to be an ongoing cellular process begun by the carcinogen exposure, presumably by reversing the carcinogen-induced change in gene expression. Thus, it is believed that a cellular process in many cells is begun by carcinogens and that certain "anticarcinogenic" protease inhibitors can turn this process off before a rare, later, mutation-like event (leading directly to the malignant state) can occur (Kennedy, 1982, 1985a,b).

High-frequency processes that lead to rare genetic events are already known to exist. For example, the "SOS" repair system, named after the international distress signal, in bacteria is known to be turned on in cells exposed to carcinogens; the induction of this system then leads to rare genetic events (mutations) (Witkin, 1976). This is not a likely mechanism to explain carcinogenesis, however. There is little or no effect of protease inhibitors on mammalian DNA repair processes (Borek and Cleaver, 1981; Korbelik

et al., 1988). In fact, there is little evidence to support the existence of a mammalian error-prone repair system similar to SOS repair in bacteria, as has been reviewed (Rossman and Klein, 1985). Furthermore, this system is not persistently activated in bacteria, as it would need to be to explain the phenomena involved in carcinogenesis. A system that does have the characteristic of persistent activation is radiation-induced recombination in yeast (Fabre and Roman, 1977). This is a system in which both X-rays and UV light have been shown to induce recombinational events that continue to occur for many generations post-irradiation. Radiation could induce such a system in mammalian cells that could then produce the transformed cell genotype.

It is reasonable to expect proteases to be involved in the induction of the systems described above. Proteases are known to play a central role in gene regulation (Gottesman, 1987). SOS functions are known to be activated by a protease (Little *et al.*, 1980) and are inhibited by protease inhibitors (Meyn *et al.*, 1977). Radiation-induced recombination in yeast is also suppressed by protease inhibitors, with inhibitors of chymotrypsin being the most effective protease inhibitors studied for suppression of the process (Wintersberger, 1984). As discussed above, it has been observed that inhibitors of chymotrypsin are also the most effective protease inhibitors at suppressing radiation-induced transformation *in vitro* (Kennedy, 1985a), a correlation that may suggest a relationship between the process operating in yeast and that involved in the induction of malignancy in mammalian cells.

An ongoing process of the sort that is envisioned as being induced by carcinogens in mammalian cells may be operating in the cells of patients with Bloom's Syndrome. It is known that patients with Bloom's Syndrome have a higher than normal rate of cancer development and that the cells of these patients are constantly generating new chromosomal abnormalities (reviewed by German, 1995). It has been observed that the anticarcinogenic protease inhibitors can reduce the levels of chromosome abnormalities in the cells of patients with Bloom's Syndrome (Kennedy *et al.*, 1984b). It is conceivable that the high-frequency cellular process hypothesized to be induced by carcinogens is continuously operating in the cells of patients with Bloom's Syndrome.

A process of the sort likely to explain the observations described above on carcinogenesis in mammalian cells has not been identified yet. There is much evidence to suggest that such a mechanism is involved in the induction of cancer in animals, however. Recently, several *in vitro/in vivo* systems have been developed in which it is possible for investigators to count the number of carcinogen-treated cells that give rise to cancer in animals. Examples of such studies have been published by Clifton *et al.* (1991), Terzaghi-Howe (1989), Ethier and Ullrich (1982), and Kamiya *et al.* (1995). In these studies, it has been observed that only a few carcinogen-treated cells are capable of giving rise to cancer in animals, as reviewed elsewhere (Kennedy, 1994); the frequencies of malignant transformation of the cells are

extremely high in such studies ($>10^{-2}$). It is known that the frequencies of single-base mutations are very low, on the order of 10^{-6}/cell/generation, as reviewed elsewhere (Kennedy et al., 1984a; Kennedy, 1994, 1996b). As the frequencies of malignant transformation are far higher than the frequencies expected for single-base mutations, results from these studies suggest that single-base mutations are not likely to be involved as the initiating events in carcinogenesis in the systems studied. While it is still widely believed that single-base mutations in certain proto-oncogenes, etc., are capable of leading to cancer (usually thought to be through a multistep process), the studies utilizing the *in vitro/in vivo* systems raise a question about whether a single-base mutation is the usual method by which carcinogens start the process that eventually leads to the malignant state.

2.2.2.2. Selective toxicity in premalignant and certain malignant cells. Toxic effects of BBI have been observed in two different types of premalignant cell types studied in the Kennedy laboratory. For these studies, effects for PBBI and BBIC are comparable; toxic effects of BBI have been observed both in premalignant cells of connective tissue origin, "10T1/2-myc" cells (Taylor et al., 1992), and epithelial cell origin, "308" cells (Strickland et al., 1988). This toxic effect of BBI in atypical cells extends to certain malignant cells. An example of one line of malignant cells that responds to toxic effects from treatment with BBI is LNCaP cells. LNCaP cells are a hormonally responsive human prostate cancer cell line (Horoszewicz et al., 1983). The cell-killing effects of BBI in LNCaP cells are reflected as inhibited growth in growth curves of cells utilizing this cell line.

The cell-killing effect of BBI in premalignant and certain malignant cells is a relatively small effect (which varies from 25 to 75% toxicity in different cell lines), but it must be remembered that this is an agent that has a selective toxicity for premalignant cells (and some malignant cells) and is nontoxic to normal cells. For tumors in which cell growth is slow (e.g., certain prostate tumors), BBI/BBIC could be considered as a cancer therapeutic agent.

In previous studies performed in 10T1/2 cells (the rodent cell system used for most studies on the suppression of malignant transformation *in vitro* by protease inhibitors), it was concluded that the anticarcinogenic protease inhibitors did not have effects on cells that were already transformed (e.g., Kennedy and Little, 1978, 1981a). For the malignantly transformed 10T1/2 cells that were studied, F-17 cells (transformed by ionizing radiation) and Cl-16 cells (transformed by a chemical carcinogen, 3-methylcholanthrene), the anticarcinogenic protease inhibitors did not have the ability to affect the growth of the cells. Further, it was concluded that the malignant 10T1/2 cells did not have an altered capacity to express themselves as malignantly transformed cells when grown in the presence of the anticarcinogenic protease inhibitors (Kennedy and Little, 1981a). This lack of effect of the anticarcinogenic protease inhibitors on F-17 and Cl-16 cell growth does not apply to all malignant cell growth, as described above. In fact, the toxic effect for some premalignant and malignant cells is highly significant and could account for the ability of the anticarcinogenic protease inhibitors to suppress malignant transformation *in vitro* or carcinogenesis *in vivo* when administered to cells in culture or experimental animals at long time periods after exposure to the carcinogenic agent(s).

Other investigators have also studied the effects of the anticarcinogenic protease inhibitors on the growth of malignant cells. From their studies, Kuroki and Drevon (1979) concluded that the anticarcinogenic protease inhibitors did not affect the growth of malignantly transformed C3H10T1/2 cells, while other studies have concluded that certain protease inhibitors can suppress the growth of malignantly transformed cells (e.g., Noonan and Noonan, 1977; Clark et al., 1993).

2.2.3. Dose-response relationship for anticarcinogenic activity (*in vivo* and *in vitro*). Much information is known about the dose-response relationship for BBI to inhibit either transformation *in vitro* or carcinogenesis in animals. It has been observed in both *in vitro* transformation (Yavelow et al., 1985; Kennedy, 1985a) and *in vivo* carcinogenesis (e.g., St. Clair et al., 1990a; Kennedy et al., 1993a) studies that concentrations or doses of PBBI and/or BBIC varying over orders of magnitude have the same suppressive effect on carcinogenesis; thus, there appears to be a "saturation" effect in the ability of BBI to inhibit carcinogenesis. The decline in the ability to inhibit carcinogenesis both *in vivo* and *in vitro* is a steep dose-response relationship over a very narrow dose range of BBI. Similar dose-response curves for the inhibition of proteolytic activity have been observed *in vitro* and *in vivo* (Billings et al., 1987; Oreffo et al., 1991) with a steep decease in proteolytic activity over a very narrow concentration range of PBBI/BBIC. At concentrations above this range, additional increases in inhibitory activity are much less pronounced or absent (e.g., Billings et al., 1987; Oreffo et al., 1991). An example of the saturation effect in the ability of BBI to inhibit carcinogenesis can be observed in the comparable abilities of PBBI to inhibit liver tumorigenesis when present at 0.1% or 0.01% of the diet (St. Clair et al., 1990a).

The dose-response relationship for BBI to inhibit carcinogenesis *in vitro* or *in vivo* is unusual in that concentrations/dose levels of BBI varying over orders of magnitude have the same suppressive effect. This phenomenon may be due to the characteristics of the target protease involved in carcinogenesis. Studies on the M_r 44-kDa protease described in Section 2.5.8.2, believed to be a target protease of the anticarcinogenic protease inhibitors, have shown that this protease must be activated with trypsin to be active (Billings et al., 1991b; Billings and Habres, 1992). It is thought that proteases such as this, requiring trypsin activation, are carefully controlled by cells and only very small amounts of these enzymes are active at a given time; thus, very low concentrations of protease inhibitors would be effective at inhibiting such a protease (Billings et al., 1991b).

2.3. Pharmacokinetic Studies of Bowman-Birk Inhibitor (in Relation to Anticarcinogenic Activity)

2.3.1. Studies in animals—with [^{125}I]Bowman-Birk inhibitor.

It previously was believed that very little of the soybean-derived protease inhibitors would be taken up into the bloodstream and distributed to organs outside the gastrointestinal (GI) tract following dietary ingestion; thus, a number of publications from Kennedy and colleagues (e.g., Persiani et al., 1991; Ekrami et al., 1993, 1995a,b; Larionova et al., 1994b) address mechanisms to increase the uptake of BBI into the bloodstream so that organs outside of the GI tract would be exposed to increased concentrations of BBI following delivery via the diet. It now appears that reasonable dietary concentrations of BBI result in a sufficient amount of BBI being taken up into the bloodstream, with subsequent distribution throughout the body, to prevent carcinogenesis at many different organ sites. Billings et al. (1992) present data demonstrating that approximately 50% of the BBI from dietary sources is taken up into the bloodstream and distributed throughout the body. BBI is an extraordinary protein, with the ability to survive the digestive process and reach the colon in an active form (Billings et al., 1992; Yavelow et al., 1983). BBI can be measured in colonic epithelial cells in an active form, in the bloodstream, and in the urine: some of the BBI recovered from the urine is capable of interacting with proteases in the same manner as expected for BBI (Yavelow et al., 1983; Billings et al., 1992).

Information about the absorption, distribution, and excretion of BBI primarily comes from animal studies utilizing radiolabelled BBI. Studies performed in the Kennedy laboratory indicate that approximately one-half of the BBI administered orally is excreted in the feces in an unaltered form, while the rest enters intestinal epithelial cells (Billings et al., 1991a) or crosses the intestinal lumen via a paracellular mechanism (Billings et al., 1992). At 3 hr after an oral [^{125}I]BBI dose, BBI is widely distributed in the body, and present in an active form in all major internal organs examined (except the brain): the percent distributions of the labelled BBI found in each organ and in body fluids have been described by Billings et al. (1992). It is known that some of the BBI excreted into the urine still possesses protease inhibitor activity (Billings et al., 1992; Yavelow et al., 1983). When [^{125}I]BBI is administered to animals by oral gavage, the calculated serum half-life ($t_{1/2}$) is 10 hr in both rats and hamsters. Data from which the serum $t_{1/2}$ have been calculated are shown in Fig. 4; the detailed methods for these studies are given in the figure legends. Several investigators have reported on the bioavailability and distribution of BBI (e.g., Persiani et al., 1991; Madar et al., 1979; Yavelow et al., 1983; Billings et al., 1992). From the many distribution studies performed with [^{125}I]BBI, it is clear that a large percentage, approximately 40–50%, of the labelled BBI is excreted via the feces. Of the 50–60% of the [^{125}I]BBI that is taken up into the bloodstream (or into intestinal epithelial cells), at 2–3 hr after administration, [^{125}I]BBI is widely distributed in the body, with 2–5% of the ingested BBI being present in the bloodstream and approximately 1–2% of the [^{125}I]BBI present in all organs examined (except the brain). From animal studies, the time for BBI to travel through the GI tract and either be taken up into the bloodstream or colonic tissue, or enter the feces, is approximately 5 hr. Once BBI has entered the bloodstream, it is cleared very rapidly. After an i.v. injection of [^{125}I]BBI, most of the labelled inhibitor is cleared from the blood by 30 min, and all of it has disappeared from the blood by 24 hr (Persiani et al., 1991). After reaching an organ, BBI appears in the epithelial cells of the organ very quickly. For example, peak concentrations of BBI appear in hamster cheek pouch epithelial cells at 15 min after BBI is injected into the cheek pouch. In these studies performed in the hamster cheek pouch, the appearance of [^{125}I]BBI in the blood correlates with its appearance in the cheek pouch epithelial cells.

Estimates have been made in efforts to relate the data collected from the biodistribution studies to potential anticarcinogenic activity. In studies in which 0.01% dietary PBBI was shown to completely prevent the induction of liver tumors in animals, it was calculated that the amount of ingested PBBI would result in 5 µg of PBBI, or 6.25×10^{-10} mol of PBBI, reaching the liver per day (St. Clair et al., 1990a). This amount of PBBI reaching liver cells is well within the range of concentrations of PBBI that have been shown to completely prevent/suppress the malignant transformation of cells in vitro (Yavelow et al., 1985). The lowest effective dose in the dose-response curve for PBBI to inhibit malignant transformation in vitro (Yavelow et al., 1985) or the induction of DMH-induced liver angiosarcomas in animals (St. Clair et al., 1990a) has not been found, but it is possible that considerably lower doses than those studied will be effective in both of these systems previously studied. These results demonstrate that the amount of PBBI reaching internal organs such as the liver after dietary ingestion of BBI is capable of preventing cancer. The concentrations of PBBI reaching the liver following dietary BBI ingestion appear to be roughly comparable with the amounts of BBI reaching other organs outside the GI tract. Specifically, biodistribution studies have been done for the breast and the prostate, and it has been concluded from these studies that when administered orally, the amount of BBI reaching these organs could be sufficient to have anticarcinogenic effects in these organs. Details of the biodistribution studies for the breast follow. To determine the amount of PBBI that would reach breast tissue, the distribution of labelled PBBI was determined in mammary tissue of female rats. In these studies, it was observed that at 3 hr, 6 hr, and 24 hr post-gavage, the amounts of [^{125}I]BBI reaching mammary and liver tissue were comparable, with increasing amounts appearing in each of these organs with increasing time post-gavage (P. C. Billings and A. R. Kennedy, unpublished data). As comparable amounts of ingested PBBI (on a per gram basis) go to liver and mammary tissue in animals, it is expected that there will be at least as great a suppressive effect observed for BBI in carcinogen-treated breast tis-
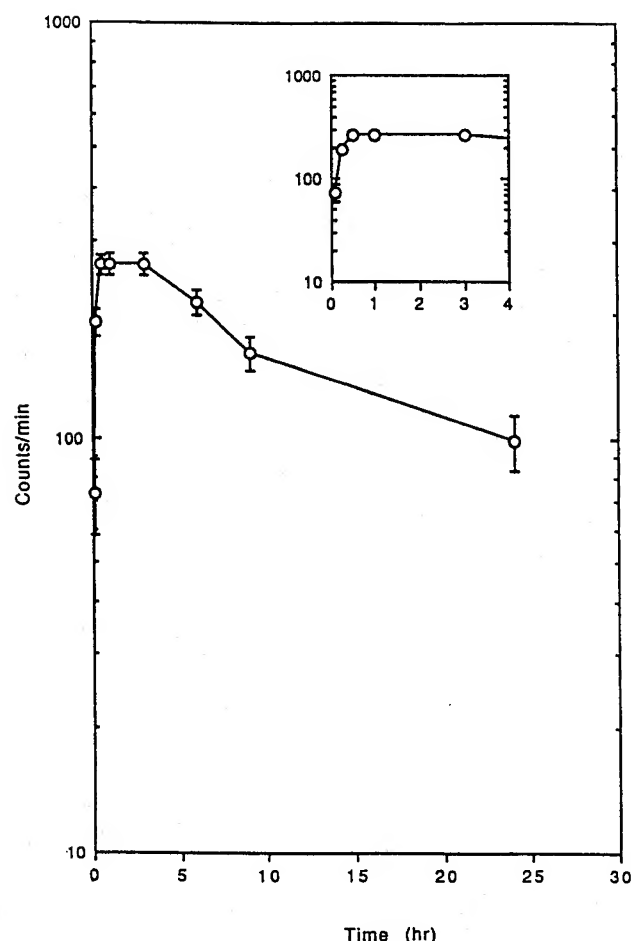
FIGURE 4. Studies on the $t_{1/2}$ of BBI in animals. These studies were performed with [^{125}I]BBI in the Kennedy laboratory in both rats and hamsters to determine the $t_{1/2}$ of BBI (P. Maki, P. C. Billings, and A. R. Kennedy , unpublished data). The following methods were utilized in these studies. (1) *Radio-iodination of BBI.* BBI was labelled with ^{125}I using iodobeads, as previously described (see Billings *et al.*, 1987). Briefly, 15 mL of BBI (1 mg/mL) was mixed with 200 mL of 0.1 M phosphate buffer (pH 6.8) in a 1.5-mL microcentrifuge tube. Two iodobeads (Pierce) were added, and the iodination reaction was initiated by the addition of 0.1 mCi of Na ^{125}I. The centrifuge tube was capped and the reaction was allowed to proceed for 1 hr at room temperature. Labelled BBI was separated from free iodine by gel filtration chromatography on a Bio-Gel G6DG column (0.7 × 10 cm) in PBS. The specific activity of the labelled BBI was 2 × 10^7 cpm/mg. The labelled BBI standard eluted from the G-25 column at fraction 9. (2) *Determination of the $t_{1/2}$ of the* BBI. Male CD rats were starved overnight. Prior to administration of [^{125}I]BBI, a blood sample was obtained by tail bleed. [^{125}I]BBI (10^6 cpm) was administered in 0.5 mL PBS by oral gavage. Blood samples (0.8 mL) were obtained by tail bleed at 5 min, 15 min, 30 min, 60 min, 3 hr, 6 hr, and 12 hr post-gavage. Additional samples were taken at 24 and 48 hr post-gavage by cardiac puncture. Blood samples were allowed to clot at room temperature, and the clot was gently separated from the sides of the microfuge tube with a wooden rod. The samples were then centrifuged at 10,000 g in a microcentrifuge for 10 min. One hundred microliter aliquots of each serum sample were assayed for the presence of [^{125}I]BBI. The amount of labelled BBI present in serum as a function of time is shown in Fig. 4. The calculated $t_{1/2}$ for BBI in rats is 10 hr. A similar procedure was

sue as previously observed for the carcinogen-treated liver. It is known that low dietary concentrations of either PBBI and BBIC have major suppressive effects on liver carcinogenesis (e.g., St. Clair *et al.*, 1990a). The effects of soybean-containing diets on breast carcinogenesis and the likely effects of the soybean-derived protease inhibitors on the suppression of breast carcinogenesis in animals are described elsewhere (Kennedy, 1995a).

2.3.2. Studies in humans—with antibodies (which react with reduced Bowman-Birk inhibitor). As part of the bioavailability/distribution studies of BBI, it initially was planned that human pharmacokinetic studies would be performed with BBI antibodies produced by Brandon *et al.* (1989). These antibodies can readily detect and measure BBI present in food samples. When PBBI is added directly to human serum samples, it can be readily detected and measured in a highly quantitative fashion by the Brandon antibodies. Unfortunately, dietary administration of BBI results in a form of BBI in the bloodstream and urine that cannot be detected with the Brandon antibodies, despite the fact that the BBI appearing in blood and urine has the same molecular weight as BBI and the ability to inhibit trypsin and chymotrypsin comparable with that of BBI. As antibodies that react with reduced BBI are necessary to detect BBI in blood and urine samples, as described by Wan *et al.* (1995), it is assumed that BBI is present in a reduced form in body fluids.

Thus, pharmacokinetic BBI data in humans are limited to studies performed with antibodies that react with reduced BBI. Using these antibodies, a pattern similar to that observed in animal studies has emerged. The human data suggest that BBI is cleared rapidly from the bloodstream, as observed in the animal studies that have been performed. For the individuals in the human/pharmacokinetic studies, the concentrations of BBI in the urine peaked within 1–6 hr after an oral BBI dose, with urinary concentrations of BBI returning to baseline levels by 24 hr following BBI dosing (X. S. Wan, L.-J. W. Lu, K. E. Anderson and A. R. Kennedy, unpublished studies). Pharmacokinetic studies of BBI have been carried out in rodents, dogs, and humans with antibodies that react with reduced BBI (Wan *et al.*, unpublished data).

2.4. Potential Toxicity of Bowman-Birk Inhibitor/Bowman-Birk Inhibitor Concentrate

The soybean protease inhibitors have been viewed in the past as toxic agents in soybeans, with the potential to inhibit the growth of young animals, and, perhaps, to contribute to pancreatic cancer development, as described in detail in Section 5.2. It is now recognized by many investigators

utilized to determine the $t_{1/2}$ in hamsters. Similar results were obtained, and the calculated half-life for BBI in hamsters was determined to be 10 hr.

that the soybean protease inhibitors are not responsible for the growth-suppressing effects of raw soybean products in young animals, as has been reviewed elsewhere (Kennedy, 1993a; Birk, 1993). The effect on the promotion of atypical growth in the rat pancreas, which has been previously associated with the soybean protease inhibitors, is not expected to occur in humans, as has been discussed elsewhere (e.g., Kennedy, 1993a, 1994, 1995a, 1996a; Kennedy et al., 1993b). The potential effects of the soybean protease inhibitors on the rat pancreas are triggered by the ability of the protease inhibitors to inhibit trypsin, but not chymotrypsin (Birk, 1976, 1985, 1993), while the ability to inhibit carcinogenesis is associated with the ability to inhibit chymotrypsin (which is why the strength of BBIC doses is measured in CI units) (Yavelow et al., 1985; Kennedy et al., 1993b). (Thus, the two protease inhibitor sites, for trypsin and chymotrypsin, in BBI are separable and distant from each other in the molecule, as can be readily observed in Fig. 1.) As described in Section 2.1, compared with raw soybeans, the TI activity of BBIC has been greatly reduced and the chymotrypsin inhibitory activity has been greatly increased (Kennedy et al., 1993b). BBIC has been treated like a drug by the FDA. When tested in animals at doses that are up to 2 orders of magnitude higher than the doses of BBIC being utilized in humans, BBIC does not lead to pathologic effects in any organ, including the pancreas, in 3 species of animals (mice, rat, hamsters) studied in the Kennedy laboratory (reviewed in Kennedy, 1993a; Kennedy et al., 1993b) and in two species of animals (rats, dogs) studied at the Southern Research Institute (SRI) in Birmingham, AL (USA) (J. Page et al., unpublished data). Animal experiments at these high doses of BBIC have been carried out for as long as the animals' life spans in the Kennedy laboratory and for 3 months in dogs and rats at SRI. Thus, even at these extremely high doses of dietary BBIC, no histopathologic alterations in the pancreas have been observed. On a comparable weight basis, the doses of BBIC needed to prevent cancer development are well below the doses of soybean protease inhibitor activity that might be expected to lead to pancreatic abnormalities in rats (Kennedy, 1993a, 1995a; Kennedy et al., 1993b). Thus, even on a theoretical basis, the doses of BBIC being utilized for the prevention of cancer in humans should not be a problem for the human pancreas. Adverse histopathologic effects of BBI were not observed in other organs either as part of the studies performed at SRI, as described in Section 2.4.1.

2.4.1. Summary of the potential toxicological effects of Bowman-Birk inhibitor/Bowman-Birk inhibitor concentrate in animals and in vitro.

BBI/BBIC has been extensively studied in in vitro systems (Yavelow et al., 1983, 1985; Kennedy, 1985a; Baturay and Kennedy, 1986; Kennedy, 1984); they have no toxic effects in any normal cells studied, but do have toxic effects in premalignant and some malignant cells, as described in Section 2.2.2.2. In animal studies conducted in the Kennedy laboratory extending as long as the animals' normal life spans (and up to a dose as high as 1% dietary BBIC), no adverse biological effects were observed in the treated animals (as reviewed by Kennedy et al., 1993b). As part of these studies, body weight and the pancreas/body weight ratio were determined in all studies involving animals treated with BBI (and other protease inhibitors); protease inhibitor treatment did not alter these parameters in studies designed to evaluate its ability to serve as a cancer preventive agent when administered to animals at a nontoxic dietary level, as has been reviewed elsewhere (Kennedy et al., 1993b; Kennedy, 1993a). Histopathological analysis of the pancreata of the animals has shown no effects due to protease inhibitor treatment in any of the experiments involving long-term treatment of animals with BBI, as has been reviewed (Kennedy et al., 1993b). It is of interest that in studies on the anti-teratogenic effects of BBI (von Hofe et al., 1990) and in several studies on the suppression of lung tumorigenesis by BBI (Witschi and Kennedy, 1989), BBI was injected into animals (i.p.) with no apparent problems. [As part of lung carcinogenesis studies, many i.p. injections of BBI were given over periods of several weeks, and no toxicity was apparent (Witschi and Kennedy, 1989)]. Thus, the only toxic effect observed in all of the studies performed in the Kennedy laboratory was a toxic effect on the developing embryo when BBI was injected at a high level into pregnant animals. These studies are described in Section 2.5.4. In summary, both PBBI and BBIC have anticarcinogenic activity at nontoxic levels in vivo.

In support of human cancer prevention trials with BBIC, subchronic oral toxicity studies of BBIC in dogs and rats were conducted for the Chemoprevention Branch, National Cancer Institute, at SRI under the supervision of Dr. John Page, Study Director. On the basis of these studies, multiple dosing protocols were approved by the FDA as part of INDs 34671 and 52,642 (studies in humans have involved BBIC trials at 25–800 CI units/day, for a total of 6 months of BBIC administration). Details of these animal studies are as follows. Rats were dosed once daily with BBIC suspended in aqueous carboxymethyl cellulose at a vol of 5 mL/kg body weight/day; dogs were dosed once daily with neat BBIC in gelatin capsules. These studies in rats and beagles showed that doses as high as 1000 mg/kg body weight/day had no effects on the parameters measured, which included survival, feed consumption, body weight, EKG, ophthalmic examination, hematology, clinical chemistry, urinalysis, organ weights, and gross and microscopic pathology. The conclusions of the standard toxicity and pathology evaluations were that in rats, there were no treatment-related adverse effects. In the dogs, the only potential treatment-related effect was that of sporadic incidences of diarrhea, mainly in male dogs. This was the only clinical sign considered to be potentially treatment related. In the study, it was not clear that the diarrhea was treatment related; however, as noted by the study director, ". . . if treatment-related, the diarrhea was not severe enough to alter body weight, feed consumption, or serum electrolyte concentrations." Given the potential effect of diarrhea from

BBIC, the high dose (1000 mg/kg body weight/day) was considered to be the maximum tolerated dose for dogs. It is thought that the (potential) sporadic diarrhea in male dogs was due to the sheer bulk of material (BBIC) ingested by the dogs at the maximum tolerated dose of 1000 mg/kg body weight/day. The conclusions from these studies were as follows: In 90-day subchronic studies in rats and dogs, BBIC did not elicit significant toxicity at doses of 100, 500, and 1000 mg/kg body weight. The no-observed adverse effect level in rats was >1000 mg/kg body weight and the maximum tolerated dose in dogs was 1000 mg/kg (James A. Crowell, personal communication).

2.5. Known Biological Effects of Bowman-Birk Inhibitor

2.5.1. Potent protease inhibitor. BBI is known to inhibit the proteolytic activity of several well-characterized proteases, including trypsin (Birk, 1976), chymotrypsin (Birk, 1976), cathepsin G (Larionova et al., 1994a), elastase (Larionova et al., 1994a), and chymase (Ware et al., 1997). Despite the ability to inhibit the activity of these proteases, BBI does not have detectable effects, at the doses studied in animals, on the biological functions in which these proteases are known to play important roles (reviewed by Kennedy, 1993a).

2.5.2. Anti-inflammatory activity of Bowman-Birk inhibitor. It is now known that BBI is highly anti-inflammatory. In several of the animal carcinogenesis studies, a reduction in the level of lymphoid aggregates or lymphocytic infiltrates in the BBI/BBIC-treated animals was directly correlated with a decrease in tumor yields (e.g., von Hofe et al., 1991; St. Clair et al., 1990a), as illustrated in Fig. 5.

The mechanism by which BBI reduces the inflammatory response is unknown, although several different reactions could contribute to this activity. As one example, it is known that BBI is a potent inhibitor of mast cell chymase (Ware et al., 1997). Chymase is known to perform a number of pro-inflammatory functions, including the activation of procollagenase (Saarinen et al., 1994) and the activation of the inactive cytokine, 31 kDa interleukin 1β to the 18-kDa biologically active species (Mizutani et al., 1991). It is thought that the conversion of the 31-kDa inactive cytokine to an active cytokine has a critical role in the initiation of the inflammatory response (Mizutani et al., 1991). The anticarcinogenic protease inhibitors have been shown to prevent the influx of polymorphonuclear leukocytes (PMN) into regions of inflammation, as well as the production of active oxygen species by these cells (as discussed by Goldstein et al., 1979; Witz et al., 1980; Troll et al., 1982, 1984; Frenkel et al., 1987; Frenkel, 1992), which would be expected to contribute to anti-inflammatory activity.

The anticarcinogenic protease inhibitors previously have been shown to inhibit inflammation specifically associated with tumor promotion (Goldstein et al., 1979; Witz et al., 1980; Troll et al., 1982), which is a stage of carcinogenesis subject to inhibition. Thus, the inhibition of promotion by protease inhibitors may be viewed as a beneficial effect. Thus far, no investigators have shown effects of BBI on the normal functioning of cells of the immune system (Goldfarb et al., 1989; Maki and Kennedy, 1992; Maki et al., 1994).

2.5.3. Prevention of hair and weight loss. In a study designed to determine whether the dietary addition of BBIC could inhibit radiation-induced leukemogenesis in C57BL mice, three unexpected findings were noted. At the time of animal sacrifice or death due to leukemia, BBIC-supplemented mice looked healthier in that they had shinier coats, they had no radiation-induced hair loss, and they weighed more than the non-BBI-supplemented mice; some of the findings from these studies have been published (Evans et al., 1992; Kennedy, 1993d). In addition to looking healthier, the BBIC-supplemented mice appeared to have a higher quality of life than the non-BBIC-supplemented mice. The BBIC-supplemented mice looked like normal animals, while the non-BBIC-supplemented mice were observed to be in crouched positions much of the time. Representative photographs of the irradiated mice with and without BBIC dietary supplementation are shown in Fig. 6.

In this study, it was assumed that the hair loss was due to irradiation of the animals. Hair loss as a part of radiotherapy for tumors has been studied extensively. When hair loss is directly due to the radiation exposure, it is usually irreversible. Most of the studies that have been performed to minimize radiation dermatitis and resultant hair loss have been aimed at minimizing local radiation-induced inflammation (Kim et al., 1983; Verhey and Sedlacek, 1983; Liebner et al., 1962; Potera et al., 1982; Ohlsen et al., 1987). This radiation-induced inflammation is thought to be mediated by mast cells (Swartz and Austen, 1984). The success of the previously used therapies has been minimal, and none are in common clinical usage. The hypothesis in these experiments was that BBI could modify coat quality and hair loss either by locally affecting mast cell-modulated inflammation or by acting upon a systemic effector, such as cachectin.

Radiation doses below 3 Gy have been associated with skin connective tissue changes (Eisen and Wilson, 1957; Kelenyi, 1953); mast cells within the connective tissue are known to release a variety of proteolytic enzymes in the local tissue environment that degrade ground substance. Chemotactic mediators are also released from the mast cell granules and are thought to be important for the local inflammatory response in skin (Swartz and Austen, 1984); these chemotactic mediators eventually lead to late radiation-induced skin changes.

In this study, it was observed that the animals looked healthier in that they maintained their weight, even when they had lymphosarcomas/leukemia. From the results of this study, it has been hypothesized that the anticarcinogenic protease inhibitors had an effect on cachexia due to the malignancy. Cachectin is a macrophage-derived hormone that has been shown, when administered at low doses over
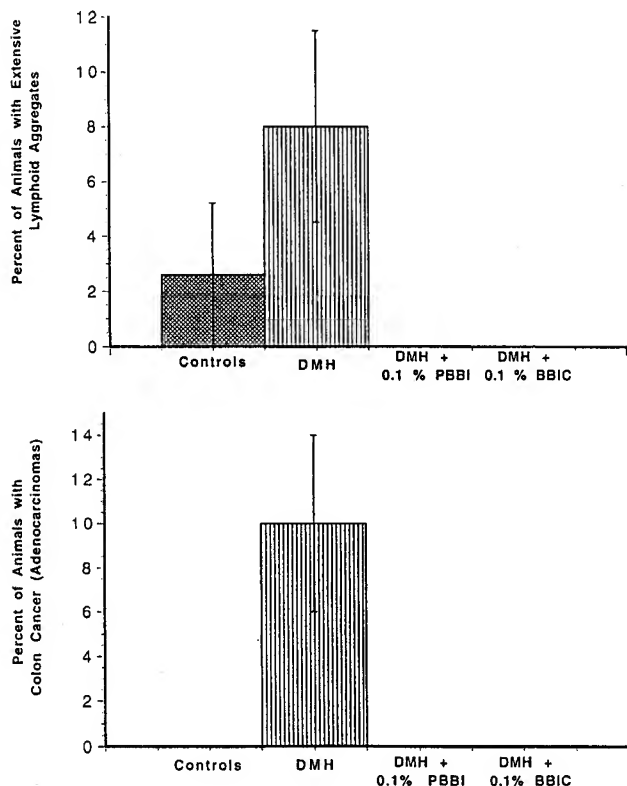
FIGURE 5. (top) Anti-inflammatory activity of PBBI/BBIC. BBI has been shown to have anti-inflammatory activity in several different organ systems; the results for the colon are shown. DMH is an inflammatory agent with the ability to cause the accumulation of "extensive lymphoid aggregates" in the colons of treated animals. When animals are treated with BBI along with DMH, there is a marked reduction in numbers of extensive lymphoid aggregates. (bottom) Suppressive effects of PBBI/BBIC on colon carcinogenesis. The anti-inflammatory activity of BBI parallels its ability to serve as a cancer chemopreventive agent for colon carcinogenesis. Details of this study are given in St. Clair et al. (1990a).

a prolonged period of time, to induce a state of anorexia and wasting similar to that observed in chronic infection and cancer (Beutler et al., 1985; Beutler and Cerami, 1988). It is hypothesized that in these studies, BBI inhibits the activation of cachectin by blocking the protease cleavage known to be necessary in the processing and secretion of cachectin. This hypothesis is based on the observation that BBI has been shown to inhibit a secretory process, transcytosis, as measured in MDC kidney cells (Shen et al., 1990), and has been shown to inhibit a particular protease whose characteristics suggest that it may be involved in protein processing (Billings et al., 1987).

2.5.4. Prevention of radiation-induced exencephaly. In studies designed to determine whether BBI or other anticarcinogenic protease inhibitors could affect teratogenesis, it was observed that these agents could prevent some birth abnormalities and have no effect on the incidence of other
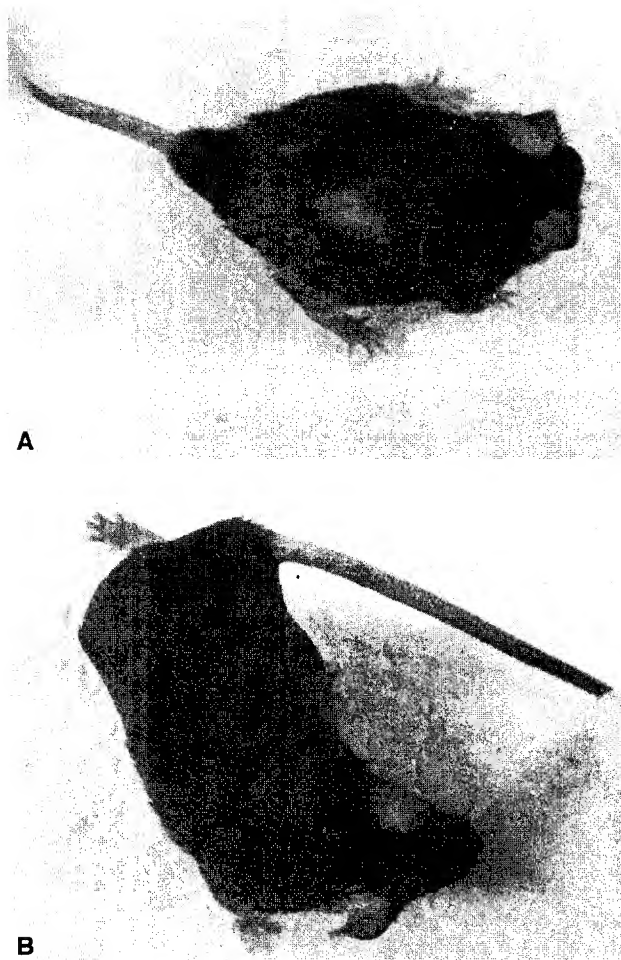
FIGURE 6. In a study of radiation-induced lymphosarcoma in mice, almost all of the irradiated animals developed the disease (86% of the animals had evidence of radiation-induced lymphosarcoma). The external appearance of the animals was markedly different, however. The BBI-supplemented mice looked healthier in that they had shinier coats, had no radiation-induced hair loss, and weighed more than the non-BBI-supplemented mice. (A) Hair loss observed in non-BBIC-supplemented mice. (B) Lack of hair loss in BBI-supplemented mice. Reproduced from Kennedy (1993d), with permission of the copyright holder, Cancer Treatment Research Foundation, Arlington Heights.

birth defects (von Hofe et al., 1990). In these studies, a high level of either antipain or BBI was shown to be toxic to the developing fetus when injected into pregnant animals; this toxic effect was measured as a reduced litter size. In these studies in which toxicity was observed for the developing fetus, there was no apparent effect on the pregnant mice themselves. In these studies, it was observed that nontoxic levels of the anticarcinogenic protease inhibitors, including BBI, were particularly effective at inhibiting radiation-induced exencephaly, a neural tube defect.

While it was observed in these studies that a high level of BBI was toxic to a developing fetus when injected into pregnant animals, this effect is not expected from BBI administered to animals via the diet. There is a clear differ-

ence in BBI effects when given p.o. vs. i.p. For example, when given via the diet, BBI is not antigenic, while BBI is antigenic when injected (Maki *et al.*, 1994). The effect observed in animals is not expected to be a problem for humans, as Asian populations with high levels of dietary protease inhibitors clearly are capable of reproducing themselves.

It is worth emphasizing that no teratogenic effects were observed for BBI in these studies; only the prevention of birth defects was observed for BBI injected into pregnant animals. The toxic effect observed, measured in terms of reduced litter size, is a common effect. Many compounds are capable of causing death to a developing embryo, resulting in a spontaneous miscarriage. This effect previously has been observed in animals for various substances at levels comparable with those representing normal human exposure levels; examples of such substances include therapeutic doses of aspirin and relatively small amounts of alcoholic beverages [less than those that lead to unacceptable blood levels of alcohol for drivers (Brent, 1978)]. Because women of child-bearing age regularly consume BBI in their normal diets, there is not likely to be an embryotoxic effect from the dietary levels of BBIC such as those that have been proposed for use in cancer prevention. From a public health perspective, the potential toxic effect of BBI on a developing embryo is far less worrisome than a potential teratogenic effect. Because the embryo is lost during a spontaneous miscarriage (usually not noticed by the pregnant female), it does not survive to become a living abnormal organism. There is no evidence to suggest that BBI/BBIC has a teratogenic effect in animals.

2.5.5. Increases life span.
In animals maintained on 1.0% dietary BBIC for their entire life span, a life-lengthening effect was observed (Kennedy *et al.*, 1993b). While laboratory animals do get various types of cancer, the usual cause of death was not cancer in the laboratory mice utilized in these life-span studies (Kennedy *et al.*, 1993b). These results suggest that the anticarcinogenic protease inhibitors such as BBI are affecting other parameters playing an important role in life span.

2.5.6. Enhances cytotoxicity of cis-platinum as a chemotherapeutic agent and serves as a radioprotector for normal cells *in vitro*.
In experiments performed to determine whether BBI might interfere with the cell-killing effects of cancer therapeutic agents, BBI was observed to potentiate the cell-killing effects of cis-platinum in human lung cancer cells and to serve as a radioprotective agent for normal cells; results of these studies are described in detail elsewhere (Kennedy, C. W. *et al.*, 1996). Dittmann *et al.* (1995) have also reported that BBI can serve as a radioprotective agent.

2.5.7. Effects on smooth muscle (urethra and corpus cavernosum).
Experiments were performed to determine whether BBI or BBIC would have direct or indirect influ-

ences on the responses of isolated strips of rabbit bladder base-urethra and corpus cavernosum to various forms of stimulation and relaxation.* The results of these studies showed that BBI and BBIC had identical properties and significant effects on the contractile and relaxant responses of the urethra and corpus cavernosum to various forms of stimulation. The results of these studies are summarized as follows: (1) BBI induced a shift to the right (inhibitory) of the dose-response curves to phenylephrine for both the proximal urethra and the corpus cavernosum. BBI had no effect on the maximal response to phenylephrine for both tissues. (2) BBI significantly inhibited neurohumoral release and subsequently stimulated contraction of the urethra. (3) BBI significantly inhibited carbachol-stimulated contraction in the urethra and carbachol-stimulated relaxation (mediated by release of nitric oxide from the endothelium) in the corpus cavernosum. (4) BBI had inhibitory effects only on low-frequency field-stimulated relaxation of the corpus cavernosum, and no effect on field-stimulated relaxation of the prestimulated urethra. These results suggest significant physiological effects on micturition and on erectile function. Specifically, the BBI effects would be expected to relax the urethra and improve flow in the presence of increased resistance, as occurs in response to benign prostatic hyperplasia, and to inhibit corporal smooth muscle tone, which would result in improved erection.

2.5.8. Effects of the anticarcinogenic protease inhibitors that are closely correlated with the prevention of cancer.
2.5.8.1. *Effects on gene expression.* It is clear that the anticarcinogenic protease inhibitors have powerful effects on gene expression, as has now been observed by numerous investigators (e.g., Chang *et al.*, 1985, 1990; Chang and Kennedy, 1988, 1993; Li *et al.*, 1992; Garte *et al.*, 1987). Early studies in this area of research showed that anticarcinogenic protease inhibitors, including BBI, reduce the levels of c-myc expression in carcinogen-treated cells (Chang *et al.*, 1985, 1990; Chang and Kennedy, 1988, 1993; Li *et al.*, 1992). While the anticarcinogenic protease inhibitors clearly affect c-myc expression in C3H10T1/2 cells, they do not affect c-myc expression in C3H10T1/2 cells that have been malignantly transformed (Chang *et al.*, 1990). This aspect of the protease inhibitor effect on c-myc expression is thought to be of central importance since the development of malignancy involves some phenomenon that can be regulated in normal cells, but is not capable of being regulated in cancer cells. C-myc expression appears to have these characteristics: it can be regulated by (anticarcinogenic) protease inhibitors in nontransformed cells and cannot be regulated in the same fashion in transformed cells. It is believed that the anticarcinogenic protease inhibitors are affecting one or more endpoints central to the process involved in malignant transformation.

---

*Malkowicz, S. B., Liu, S.-P., Kennedy, A. R. and Levin, R. M. Effect of BBI on the responses of the rabbit corpus cavernosum and urethra to specific forms of stimulation and relaxation. Manuscript in preparation.

As part of the studies on c-myc expression, it was observed that the protease inhibitors did not affect normal levels of c-myc in cells, but were only capable of returning carcinogen-elevated levels of c-myc expression to normal levels. The *in vitro* effects of the anticarcinogenic protease inhibitors on c-myc expression were also observed *in vivo*. In the irradiated colon, c-myc RNA levels are increased, but in the irradiated mouse colon exposed to BBI, the c-myc RNA levels are comparable with those observed in unirradiated colonic epithelial cells, as they are to the c-myc RNA levels in colonic epithelial cells exposed only to BBI (St. Clair *et al.*, 1990b).

A potential model by which the anticarcinogenic protease inhibitors could be affecting c-myc expression is illustrated in Fig. 7. In this model, it is assumed that radiation and chemical carcinogens are inducing proteolytic activity involved in the regulation of c-myc. This postulated protease is one that is capable of destroying a regulatory protein involved in the regulation of c-myc. It is assumed that this regulatory protein would bind to the promoter region of the gene, as illustrated in Fig. 7. It is hypothesized that carcinogen treatment would lead to increased levels of the protease, which would then lead to decreased levels of the regulatory protein; decreased binding of the regulatory protein to the promoter region of c-myc would then lead to increased levels of c-myc expression. Evidence in support of this proposed model comes from experiments showing that (1) carcinogens induce elevated levels of proteolytic activity (Kennedy and Billings, 1987; Billings *et al.*, 1987; Messadi *et al.*, 1986), as described in Section 2.5.2.; (2) radiation increases c-myc expression (St. Clair *et al.*, 1990b); and (3) c-myc gene expression increases in radiation-induced tumors *in vivo* (Sawey *et al.*, 1987).

It is conceivable that the anticarcinogenic protease inhibitors could then inhibit the protease that destroys the regulatory protein. In fact, anticarcinogenic protease inhibitors have been shown to reduce the elevated levels of carcinogen-induced proteolytic activities *in vivo* (e.g., Messadi *et al.*, 1986) and *in vitro* (e.g., Kennedy and Billings, 1987; Billings *et al.*, 1987), as well as radiation-induced c-myc levels *in vivo* (St. Clair *et al.*, 1990b; St. Clair and St. Clair, 1991).

Hypotheses for the mechanisms involved in the regulation of the c-myc gene, and the manner in which the anticarcinogenic protease inhibitors could affect those mechanisms, are described elsewhere (e.g., Chang *et al.*, 1990; Chang and Kennedy, 1993).
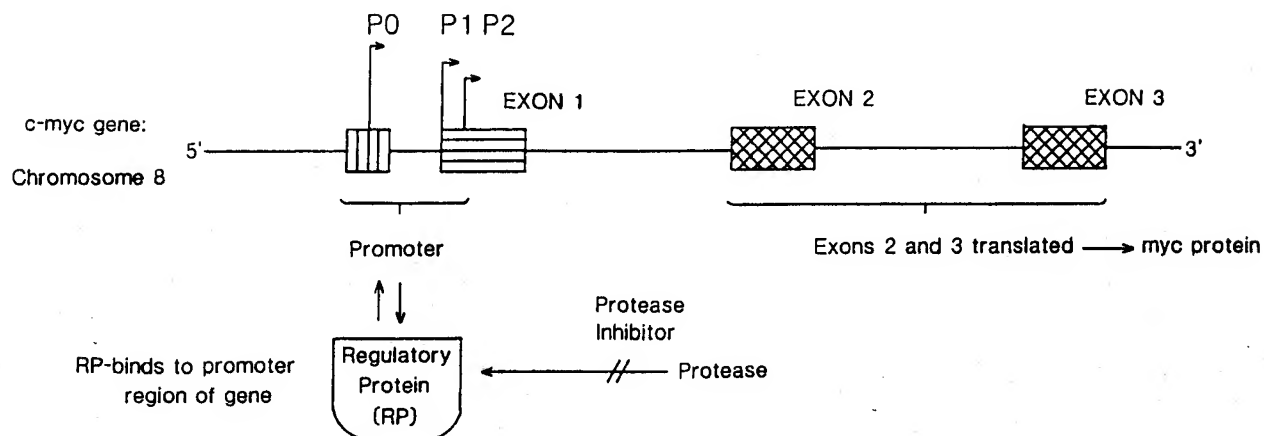
There is now much evidence that the anticarcinogenic protease inhibitors, such as BBI, affect proto-oncogene expression in many different *in vitro* systems (Chang *et al.*, 1985, 1990; Chang and Kennedy, 1988, 1993; Li *et al.*, 1992; Garte *et al.*, 1987), as well as in *in vivo* systems (St. Clair *et al.*, 1990b; St. Clair and St. Clair, 1991). A number of different proto-oncogenes and oncogenes have been studied as part of the experiments in this area of research (including c-fos, c-myc, c-erb B, c-H-ras, etc.), but the work showing the ability of BBI to affect the expression of c-myc and c-fos is most noteworthy. It is of particular interest that BBI has been shown to regulate the expression of both c-myc and c-fos *in vivo* in the irradiated mouse (St. Clair *et al.*, 1990b) and irradiated rat (St. Clair and St. Clair, 1991) colon.

It has been assumed that cooperation between increased levels of c-myc and other activated oncogenes is necessary to result in the malignant transformation of cells and that this cooperation cannot occur in the presence of the anticarcinogenic protease inhibitors that suppress the abnormally high levels of c-myc expression. Evidence has been presented in support of a role for cooperating oncogenes in the induction of transformation *in vitro* (Land *et al.*, 1983a,b).

Another way that protease inhibitors could be affecting gene expression in carcinogen-treated cells is through an effect on gene amplification. It is known that gene amplification is induced by carcinogens in a widespread fashion in mammalian cells (Lavi, 1981, 1986). It has been observed that certain anticarcinogenic protease inhibitors, including BBI, can inhibit this carcinogen-induced gene amplification (Heilbronn *et al.*, 1985; Flick and Kennedy, 1991). Radiation is known to induce gene amplification (Lavi, 1986; Tlsty *et al.*, 1984), and the radiation induction of gene amplication can be further potentiated by tumor promoting agents such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Tlsty *et al.*, 1984). The anticarcinogenic protease inhibitors have been thought to be highly antipromotional in their effects. It is expected that the mechanism(s) by which tumor promoting agents enhance malignant transformation will be directly affected by the anticarcinogenic protease inhibitors.

2.5.8.2. *Effects on protease activity.* The most direct method of determining the anticarcinogenic mechanism of protease inhibitors is to identify and characterize the proteases with which they interact. Only a few proteases have been observed to interact with the anticarcinogenic protease inhibitors; the characteristics of these proteases have been described in detail elsewhere (Billings, 1993; Billings and Habres, 1992; Billings *et al.*, 1987, 1988, 1990a, 1991b; Carew and Kennedy, 1990; Yavelow *et al.*, 1987, 1993; Messadi *et al.*, 1986; Kennedy and Billings, 1987; Habres and Billings, 1992). Proteases have been identified by both substrate hydrolysis and affinity chromatography. Utilizing substrate hydrolysis, the ability of cell homogenates to cleave specific substrates was examined, and the ability of various protease inhibitors to affect that hydrolyzing ability was determined. Utilizing affinity chromatography, specific proteases directly interacting with BBI on a BBI affinity column were identified. The Boc-Val-Pro-Arg-MCA hydrolyzing activity (Billings *et al.*, 1987; Messadi *et al.*, 1986) and the Suc-Ala-Ala-Pro-Phe-AMC (Carew and Kennedy, 1990; Yavelow *et al.*, 1987, 1993) hydrolyzing activity were identified by substrate hydrolysis, and a 44-kDa protease (Billings *et al.*, 1988, 1991b; Billings and Habres, 1992; Kennedy and Billings, 1987) has been identified by affinity

FIGURE 7. Postulated model showing interaction between a protease, anticarcinogenic protease inhibitors, and c-myc expression. Evidence in support of proposed model: 1, c-myc expression increases in radiation-induced tumors *in vivo* (Sawey *et al.*, 1987); 2 and 3, protease levels increase in carcinogen-treated tissue *in vivo* (Messadi *et al.*, 1986); and anticarcinogenic protease inhibitors return protease levels to normal (Messadi *et al.*, 1986). This model is described in more detail in the text and in Kennedy (1991). Reproduced from Kennedy (1991), with permission of *Environmental Health Perspectives* and the copyright holder, U.S. Printing Office, Washington, DC.

chromatography. The functions of these proteases are not known. As the Boc-Val-Pro-Arg-MCA hydrolyzing activity has characteristics similar to specific proteases known to be involved in growth factor processing, it has led to speculation that it has a similar function (Billings *et al.*, 1987). There is now direct evidence that BBI affects the processing of a growth factor needed for malignant cell growth, and this effect is correlated with the inhibition of growth in human small-cell-lung cancer cells (Clark *et al.*, 1993). Many types of studies have been performed by several investigators to determine the identities and functions of the proteases that are thought to be the target enzymes for the anticarcinogenic protease inhibitors (Yavelow *et al.*, 1987, 1993; Carew and Kennedy, 1990; Billings, 1993).

The levels of these different types of proteolytic activities are affected in various systems of carcinogen-exposed tissue. Following the exposure of cells to radiation and/or chemical carcinogens in a variety of different cell systems, using normal or normal-appearing cells of fibroblast or epithelial origin, increases in the levels of certain types of proteolytic activities are observed. Several different cell systems have been utilized in these studies, including 10T1/2 mouse fibroblasts (Reznikoff *et al.*, 1973), BK1 rat keratinocytes (Yuspa *et al.*, 1981), and MCF10 human breast epithelial

cells (Soule *et al.*, 1990; Tait *et al.*, 1990; Calaf and Russo, 1993; Russo *et al.*, 1993). In MCF10 cells, the levels of proteolytic activities remain elevated for as long as 6 weeks after the radiation exposure (A. R. Kennedy, unpublished data). In irradiated 10T1/2 cells, treatment with BBI reduces the elevated levels of proteolytic activities back to normal levels in parallel with the suppression of malignant transformation (measured as the yield of transformed foci of Types 2 and 3 morphology in 10T1/2 cells).

The persistent induction of proteolytic activity in carcinogen-treated tissue has also been observed *in vivo*. As one example, in buccal mucosa cells of the hamster cheek pouch, there is a persistently elevated level of proteolytic activity after exposure to DMBA, a carcinogen known to result in the induction of oral squamous cell carcinomas in the hamster cheek pouch. The Boc-Val-Pro-Arg-MCA hydrolyzing activity remains elevated months after carcinogen exposure, and treatment with BBI reduces the level of Boc-Val-Pro-Arg-MCA hydrolyzing activity back to normal in parallel with a suppression of DMBA-induced carcinogenesis in this system (Messadi *et al.*, 1986), as illustrated in Fig. 8. Elevated levels of proteolytic activities have also been observed in rat colon mucosa treated with DMH (Kennedy and Manzone, 1995). Treatment with BBI reduces the lev-

els of proteolytic activities back to normal in this system in parallel with a suppression of colon carcinogenesis (Kennedy and Manzone, 1995). The effect of BBI on the levels of proteolytic activities can be measured within 48 hr of DMH treatment (Kennedy and Manzone, 1995). The studies described above show that elevated levels of the proteolytic activities can be found in various cell types exposed to radiation or several different chemical carcinogens, suggesting that the persistent activation of proteases is a common response following cellular exposure to radiation and chemical carcinogens.

Elevated levels of proteolytic activities were also detected in human premalignant tissue and tissues at higher than normal risk of cancer development, such as in the buccal mucosa cells from patients with oral leukoplakia, smokers, and ex-smokers (Manzone et al., 1995); the "uninvolved" regions of colonic mucosa from patients with ulcerative colitis (UC); normal-appearing regions of colonic mucosa in patients who previously have had one or more adenomatous polyps removed or colon cancer (H. Manzone, G. Lichtenstein and A. R. Kennedy, unpublished data); and normal-appearing areas of breast tissue in patients with breast cancer (H. Manzone, M. Torosian, C. Powell and A. R. Kennedy, unpublished data). It is of interest that the Boc-Val-Pro-Arg-MCA hydrolyzing activity remains elevated in individuals long after they have stopped smoking (i.e., ex-smokers; H. Manzone and A. R. Kennedy, unpublished data). Thus, the Boc-Val-Pro-Arg-MCA hydrolyzing activity is persistently elevated in carcinogen-treated (i.e., exposed to cigarette smoke) or premalignant human tissue.

The studies described above suggest a general pattern that has emerged from examination of normal and carcinogen-treated cells and tissues that have or have not been exposed to the anticarcinogenic protease inhibitors. It is assumed that tissue at higher than normal risk of cancer development is similar to carcinogen-treated cells or tissue in vivo, exhibiting higher levels of proteolytic activity than normal cells/tissues, and that BBI and other anticarcinogenic protease inhibitors are able to reduce these abnormally high levels to normal levels in parallel with a reduction in risk of cancer development.

As described above, a direct approach to determining the mechanism of action of the anticarcinogenic protease inhibitors involves the isolation and characterization of the target protease(s) with which they interact. A 44-kDa protease activity that interacts with BBI, initially identified by affinity chromatography, has been characterized extensively (Billings et al., 1988, 1991b; Billings and Habres, 1992; Kennedy and Billings, 1987). It is known that this protease must be activated with trypsin and is directly inhibitable by BBI.

The 44-kDa protease that has been described appears to have significance for inflammatory processes. This protease is normally not expressed in tissue unless it has been activated with trypsin. It has been observed in an activated form (i.e., without trypsin activation) in the involved regions of the colon in patients with UC (for example, see
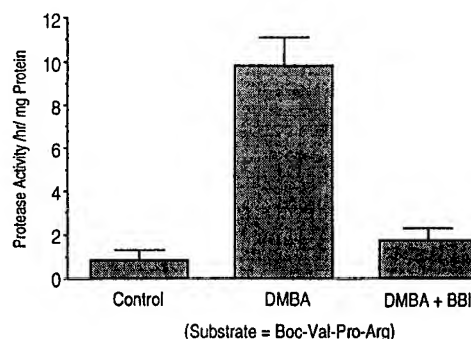

(Substrate = Boc-Val-Pro-Arg)

FIGURE 8. Suppressing effect of BBIC on levels of the Boc-Val-Pro-Arg hydrolyzing activity in the hamster cheek pouch treated with the carcinogen DMBA. DMBA leads to a persistent elevation of the Boc-Val-Pro-Arg hydrolyzing activity in hamster buccal mucosal cells. This proteolytic activity remains elevated months after carcinogen exposure in this system, as described in detail by Messadi et al. (1986). In normal-appearing areas of oral epithelium, DMBA treatment results in elevated levels of proteolytic activity; additional BBI exposure in these areas leads to normal levels of proteolytic activity. For these data collected at the times of animal sacrifices (in the 6-month carcinogenesis assay period), the levels of proteolytic activity in the normal-appearing areas of DMBA-treated hamster cheek pouch epithelium were significantly higher than those observed in controls, while those in the normal-appearing areas treated with DMBA and BBI were not significantly different from controls (when subjected to a t-test analysis). The BBI reduction in levels of proteolytic activities occurs in parallel with a suppression of oral carcinogenesis in this system (Messadi et al., 1986).

Fig. 9) (Hawkins et al., 1997), suggesting that this protease could be involved as a causative factor in the production of ulcers in UC patients. It is expected that BBI treatment will greatly reduce the activity of this protease, which appears to be expressed in an uncontrolled fashion in the involved regions of the colon in UC patients. In fact, recent data have shown that BBI does have a major inhibitory effect on the proteolytic activity/activities present in UC lesions (see Fig. 9 and Hawkins et al., 1997). BBIC has been shown to have the same inhibitory effect, as does BBI, on proteolytic activities associated with UC lesions (J. Hawkins, A. R. Kennedy and P. C. Billings, unpublished data).

It has also been observed that the uninvolved regions of colonic mucosa have elevated levels of proteolytic activities, as described above. It is known that patients with UC have an elevated risk of colon cancer development. It is expected that BBIC treatment will return the elevated levels of these proteolytic activities to normal levels in the uninvolved regions of the colon in UC patients. The types of proteolytic activities shown to be present at elevated levels in the colonic mucosa of UC patients are the same ones that have been observed to be at elevated levels in the colonic mucosa of animals exposed to the chemical carcinogen DMH. In animals exposed to DMH and BBI or BBIC, there is a reduction in the DMH-elevated levels of these proteolytic activities that parallels the BBI suppression of DMH-induced colon carcinogenesis (Kennedy and Man-

zone, 1995). Thus, it is expected that BBI treatment will control the elevated levels of proteolytic activities in the normal-appearing colonic mucosa of UC patients, and this action will reduce the risk of cancer development, as well as prevent the inflammatory processes that could ultimately lead to ulcer formation.

As for cancer prevention, there is evidence to suggest that BBIC treatment will have effects in inflammatory bowel disease (IBD). It is known that the incidence rates of IBD are very high in the United States compared with the rates observed in Japan and South American countries. (Dayal and DeLellis, 1989; Pena et al., 1991). It has been hypothesized that it is the high level of soybean products containing BBI consumed in Japan that leads to the low rates of IBD. (BBI-like compounds are found in high concentrations in all legumes, and are expected to be at high levels in many of the bean-based dishes of South American origin. Thus, the low rates of IBD in South America conceivably could result from high-level consumption of BBI-like molecules in the diet.) It is thought that the diet does play an important role in IBD (Hanauer and Baert, 1994; Das, 1991; Wargovich et al., 1988; Rudolph et al., 1995).

There is little information in the literature about the role of proteases in IBD and the use of protease inhibitors in treating IBD. Bohe and colleagues (Bohe, 1987; Bohe et al., 1986) give evidence that the level of proteases is increased in plasma and fecal extracts of patients with IBD and that there is a correlation between the levels of proteolytic activity and severity of the disease. There is only one article available in which protease inhibitors have been used to treat IBD. The serine protease inhibitor camostat mesilate was able to induce and maintain remission in two patients with refractory IBD (Senda et al., 1993).

It is believed that BBI will be more effective than camostat mesilate and less toxic; as described above, no toxicity is expected from BBI/BBIC. The lack of toxicity from BBI is somewhat surprising in that BBI has protease inhibitor activity comparable with that of diisopropyl fluorophosphate (DFP), also known as nerve gas, in UC lesions (as demonstrated in Fig. 10, the protease inhibitor activity of BBI is approximately the same as that of DFP when it was tested at a molar concentration 3 orders of magnitude lower than the molar concentration of DFP utilized in the experiments reported there). While BBI is a very potent protease inhibitor, its activity as a protease inhibitor is very specific and controlled.

The results shown in Fig. 10 are likely to be extremely important ones in helping to elucidate the mechanisms by which protease inhibitors such as BBI play a major role in the prevention and treatment of disease(s). As the enzymes inhibited by BBI are very specific, it is highly unlikely that BBI could be inhibiting the catalytic activity of all of the different proteolytic activities in the zymogram shown in Fig. 10. It is hypothesized that BBI is capable of inhibiting the catalytic activity of a protease whose activity is necessary for the activation of several different proteases, or a cascade of proteases, involved in inflammatory conditions.
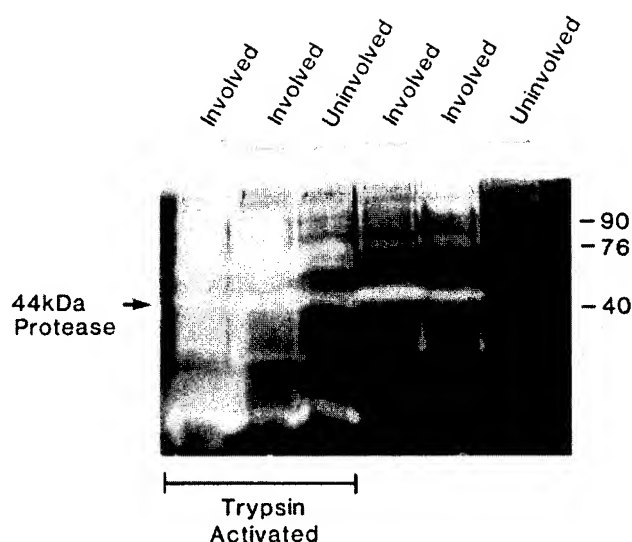
FIGURE 9. Gelatin zymogram of tissue homogenates taken from involved and uninvolved regions of the colon of a patient with ulcerative colitis. Human colonic biopsy samples were homogenized and analyzed for protease activity on gelatin zymograms. Twenty-five micrograms of protein were loaded into each lane of the gel. Samples were untreated or treated with trypsin (trypsin activated) prior to being run on the zymogram. Involved: tissue biopsy from involved region of the colon. Uninvolved: material obtained from normal-appearing areas of the colon. Note that the involved regions have higher levels of protease activity compared with the uninvolved regions. Also note the absence of the 44-kDa protease in the uninvolved sample, which was not trypsin activated (last lane on right). Numbers on right: M_r in kilodaltons. Arrow on left designates position of 44-kDa protease activity. Similar results have been obtained with three other colonic biopsy samples. Reproduced from Hawkins et al. with permission of the copyright holder, Plenum Publishing Corp., New York.

BBI is known to inhibit the activity of proteases involved in the activation of other proteases. Trypsin has been well characterized as a protease involved in the activation of other proteases (e.g., Billings et al., 1991b; Billings and Habres, 1992), and it is well established that BBI inhibits the activity of trypsin (Birk, 1976, 1985). While trypsin has been considered to be primarily a digestive enzyme, it recently has been shown to be an intracellular enzyme (Koshikawa et al., 1994; Hirahara et al., 1995). Another protease known to be involved in the activation of proteases is chymase (e.g., Saarinen et al., 1994), a proteolytic enzyme secreted from mast cells. It recently has been shown by Ware et al. (1997) that human chymase is potently inhibited by BBI. As part of these studies, it was observed that BBI was a more potent inhibitor of human chymase than any of the known physiologic protease inhibitors. It is conceivable that the secretion of chymase by mast cells triggers the activation of many proteases responsible for causing ulcers in the colons of the experimental animals in the studies described in the legend for Fig. 10. Inhibition of chy-

mase by BBI could result in the prevention of activation of proteases playing important roles in the formation of ulcers.

2.5.8.3 Effects related to free radical reactions.

It has been proposed that protease inhibitors suppress carcinogenesis by their ability to prevent free radical-induced changes in cells, as they have been shown to block free radical production in sea urchin eggs (Coburn et al., 1981) and mammalian cells. It has been shown that certain protease inhibitors prevent the influx of PMN, as well as the release of free radicals by PMN in response to TPA (Goldstein et al., 1979; Witz et al., 1980; Troll et al., 1982; Frenkel et al., 1987; Frenkel, 1992). Although BBI and other anticarcinogenic protease inhibitors have only a limited ability to deal with free radicals or their biological effects (e.g., Shasby, 1985; St. Clair et al., 1991), they can effectively achieve the same final result as antioxidants in that they can keep free radicals from being produced in cells and thereby decrease the amount of oxidative damage (Frenkel et al., 1987; Frenkel, 1992). There is clearly a strong correlation between the ability of a protease inhibitor to prevent the release of oxygen free radicals from cells and its ability to inhibit carcinogenesis, with those having CI activity such as BBI having the greatest potency (Frenkel et al., 1987; Frenkel, 1992). Recent work has indicated that BBI actually has the ability to become an antioxidant in vivo, as has been discussed elsewhere (Wan et al., 1995).

While the role of free radical-produced effects in carcinogenesis is still unclear, it is clear that the anticarcinogenic protease inhibitors can prevent the release of the superoxide anion radical and H_2O_2 from PMN, and that this effect is extremely well correlated with anticarcinogenic activity.
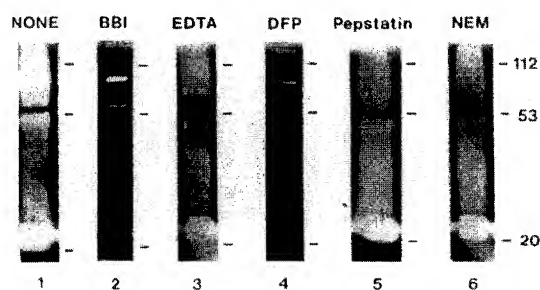

FIGURE 10. Effects of various protease inhibitors on protease activity occurring in UC lesions. Sprague-Dawley rats were treated with dinitrobenzenesulfonic (DNBS) acid to induce UC, as described by Hawkins et al. (1997). Samples of UC lesions were obtained from animals 7 days post-DNBS treatment and analyzed on gelatin zymograms. After electrophoresis, the zymograms were incubated in reaction buffer containing the indicated protease inhibitor. BBI and DFP (inhibitors of serine proteases) inhibited most of the protease activity in the samples. EDTA (an inhibitor of metalloproteases), pepstatin (an inhibitor of aspartic proteases), and NEM (an inhibitor of thiol proteases) had less effect. The lane designated "none" was incubated in the absence of protease inhibitor. Numbers on right: M_r kilodaltons. Reproduced from Hawkins et al., with permission of the copyright holder, Plenum Publishing Corp., New York.

2.5.8.4. Effects of the anticarcinogenic protease inhibitors appropriate for use as intermediate marker endpoints or biomarkers in human trials.

2.5.8.4.1. Intermediate marker endpoints. The cellular response to exposure to carcinogenic agents can be measured with intermediate marker endpoints (IME). A number of IME have been discussed as potential targets of cancer chemopreventive agents (e.g., Boone and Kelloff, 1994; Kelloff et al., 1995). Cell proliferation rates are often utilized as IME, but the anticarcinogenic protease inhibitors do not affect cell proliferation rates in normal cells or any other normal cell phenomena that have been studied (reviewed by Kennedy, 1993a). (The anticarcinogenic protease inhibitors do inhibit the growth of certain malignant and premalignant cells; in these cell types, the anticarcinogenic protease inhibitors are expected to have an effect on cell proliferation rates.) The fact that anticarcinogenic protease inhibitors do not have toxic or any other identifiable effects on normal cell functions is viewed positively, as cancer chemopreventive agents should be nontoxic in nature. While effects of protease inhibitors on endpoints in normal cells in vivo have not been observed, the anticarcinogenic inhibitors are capable of affecting several endpoints that have been altered by carcinogen exposure, the clearest examples of which are levels of proto-oncogene expression and proteolytic activity, as described in Section 2.5.8. It has been observed that carcinogen treatment increases the level of expression of these IME, and anticarcinogenic protease inhibitors return the levels to normal or background levels of activity in several of the in vivo systems studied. As examples, carcinogen treatment increases the level of c-myc gene expression in the colon (St. Clair et al., 1990b), as well as the levels of proteolytic activity in the oral epithelium (Messadi et al., 1986), while protease inhibitor (specifically, BBI/BBIC) treatment brings the carcinogen-induced, elevated levels of c-myc gene expression in the colon (St. Clair et al., 1990b) and proteolytic activity in the oral epithelium (Messadi et al., 1986) back to background levels in these in vivo systems. BBI/BBIC does not affect the endogenous levels of c-myc expression in the colon or of proteolytic activity in the oral epithelium. Levels of expression of proto-oncogenes and certain proteolytic activities currently are being utilized in BBIC human cancer prevention studies as the IME.

2.5.8.4.2. Biomarkers. Biomarkers indicate exposure to carcinogenic agents, and can also be used in cancer prevention trials as indicators of whether intervention has decreased the levels of products known to be associated with increased risk of cancer development (e.g., Lippman et al., 1990). Many of the biomarkers used are indicators of oxidative damage to DNA, as has been reviewed recently by Frenkel (1992). Protease inhibitors with chymotrypsin inhibitory activity such as BBI are known to result in decreased levels of oxidative damage to cells (Frenkel et al., 1987; Frenkel, 1992).

3. POTENTIAL MECHANISMS OF ACTION OF THE ANTICARCINOGENIC PROTEASE INHIBITORS

Although many studies have been performed to determine the mechanism for the anticarcinogenic effects of protease inhibitors, the precise mechanism(s) by which protease inhibitors suppress carcinogenesis is unknown. There are, however, several different hypotheses relating to the protease inhibitor anticarcinogenic activity that have been discussed (e.g., Kennedy, 1993a; Troll et al., 1984). Many different approaches have been taken in attempts to determine the mechanism of action of the anticarcinogenic protease inhibitors. One method of determining the mechanism of action is to study the characteristics of those protease inhibitors that are able to suppress carcinogenesis. Several different types of protease inhibitors have been shown to have anticarcinogenic activity, as has been reviewed (Kennedy, 1993a,b), and can be observed from the data summarized in Tables 1 and 2. Billings et al. (1989) have suggested that at least two different types of proteolytic activities are involved in the protease inhibitor suppression of carcinogenesis. Another approach in mechanistically oriented studies involves examination of the specific processes affected by the anticarcinogenic protease inhibitors. As examples of this method, it has been determined that BBI can inhibit the processing of endocytosed protein that is involved in transcytosis (Shen et al., 1990), can inhibit gene amplification induced by carcinogens (Flick and Kennedy, 1991), can inhibit the processing of growth factors needed for cancer cell growth (Clark et al., 1993), and can affect several other endpoints, as discussed in Section 2.5.

Unfortunately, it will not be possible to determine the actual mechanism by which any cancer chemopreventive agent has its anticarcinogenic effect(s) until the mechanism of carcinogenesis is determined with certainty. As described in Section 2.5.8., it has been hypothesized that protease inhibitors suppress carcinogenesis by affecting the levels of certain types of proteolytic activities and/or proto-oncogenes, free radical reactions, etc. Some of the other plausible mechanisms of action of the anticarcinogenic protease inhibitors are presented briefly here.

Schelp and Pongpaew (1988) have proposed that elevated levels of endogenous protease inhibitors, in particular, α_2-macroglobulin (AM), serve to protect against carcinogenesis in those with diets low in calories, fat, and animal protein, but high in vegetable content; it is thought that high levels of AM in such populations produce low cancer incidence rates for the colon, rectum, breast, and prostate. Data have been presented that indicate that AM levels do increase in response to diets that do not totally fulfill normal energy and protein requirements, as reviewed by Schelp and Pongpaew (1988). While this mechanism may operate for humans in underdeveloped countries, as suggested by Schelp and Pongpaew, it does not explain the suppression of tumorigenesis in animal experiments in which the basal diets have fulfilled normal energy and protein requirements, as, for example, in the studies by Becker

(1981), Corasanti et al. (1982), Weed et al. (1985), Messadi et al. (1986), St. Clair et al. (1990a), Witschi and Kennedy (1989), Billings et al. (1990c), von Hofe et al. (1991), and Kennedy, A. R. et al. (1993a, 1996). Thus, it is believed that other mechanisms are responsible for the suppression of carcinogenesis by protease inhibitors in the animal experiments in which the animals have been maintained on diets sufficient for their energy and protein needs.

There are many other possible mechanisms for the observed protease inhibitor anticarcinogenic activity. For example, it is reasonable to expect the anticarcinogenic protease inhibitors to affect protein kinase C (PKC). PKC is an enzyme involved in signal transduction and tumorigenesis, as it serves as the primary receptor for TPA (for reviews, see Ashendel, 1985 and Nishizuka, 1984). The binding of TPA to PKC at the diacylglycerol binding site results in activation of the enzyme (for a review, see Nishizuka, 1984). It is known that limited proteolysis of PKC with calpain converts the enzyme into a permanently active form of the enzyme, called protein kinase M (Inoue et al., 1977; Takai et al., 1977). The activity of calpain can be inhibited by high concentrations of several different thiol protease inhibitors (Ishiura, 1981; Solomon et al., 1985; Sugita et al., 1980; Takai et al., 1979). A correlation does not exist, however, between anticarcinogenic activity and the ability of a protease inhibitor to inhibit the activity of calpain. For example, some protease inhibitors lacking anticarcinogenic activity, such as tosyl-lysine chloromethyl ketone, inhibit calpain, while other protease inhibitors that do have anticarcinogenic activity, such as antipain and BBI, do not affect calpain, even when evaluated at very high concentrations (Solomon et al., 1985). Studies performed by Su et al. (1991) suggest that protease inhibitors do not suppress promotion in vitro through effects on PKC.

While the actual mechanisms involved in the protease inhibitor anticarcinogenic effects are unknown, these agents have the ability to make various endpoints altered by carcinogen exposure return to a normal state, as reviewed in Section 2.5.8. Many different endpoints altered by carcinogen exposure in cells could be playing a role in the various stages of carcinogenesis, and the return of these many endpoints to a normal state could influence carcinogenesis in a variety of ways. There are data to suggest that BBI is capable of having effects similar to several different classes of cancer chemopreventive agents. As examples, BBI has some antioxidant activity [as antibodies to reduced BBI are necessary for the detection of BBI in vivo (Wan et al., 1995)], and it is capable of altering patterns of gene expression, as reviewed in Section 2.5.8. Many different cancer chemopreventive agents have anti-inflammatory activity: BBI is known to be highly anti-inflammatory and has effects on arachidonic acid metabolism similar to the effects observed for other cancer chemopreventive agents, as discussed elsewhere (Billings et al., 1994). Billings et al. (1994) have suggested the hypothesis that the relative levels of 6-keto-PGF$_{1\alpha}$ are important in the induction of malignant transformation in vitro and that agents capable of lowering

the levels of this eicosanoid, such as BBI and other anticarcinogenic protease inhibitors, can serve as cancer chemopreventive agents. All or a combination of these different mechanisms of action could contribute to the anticarcinogenic effects of protease inhibitors.

It is likely that several different mechanisms are involved in the prevention of carcinogen-induced transformation *in vitro* by the anticarcinogenic protease inhibitors. It has been hypothesized that the first step in transformation involves the activation of early response genes, such as c-fos and c-myc, and it is known that BBI and other anticarcinogenic protease inhibitors have major suppressive effects on the expression levels of these proto-oncogenes in both *in vitro* and *in vivo* systems (Chang et al., 1985, 1990; Chang and Kennedy, 1988, 1993; Li et al., 1992; Caggana and Kennedy, 1989; Garte et al., 1987; St. Clair et al., 1990b; St. Clair and St. Clair, 1991). The gene products of both c-myc and c-fos are thought to be involved in the activation of other genes. The *c-fos* gene is considered to be a "master switch" that is part of the primary genomic response to stimulation by many different extracellular signals; these signals are then transduced through regulation of a number of secondary genes (Lucibello and Muller, 1991). Since the induction of *c-fos* gene expression precedes the activation of c-myc expression (Muller et al., 1984) and enhanced *c-fos* gene expression is known to result in the activation of *c-myc* gene expression (Su et al., 1995), it is assumed that the effect of carcinogens and BBI treatment on *c-myc* gene expression could result from the altered *c-fos* expression. The c-Fos protein, the *c-fos* gene product, is known to be involved in the up-regulation of several proteases, such as collagenase (Schonthal et al., 1988; Angel et al. 1987) and stromelysin (Matrisian et al., 1986), a neutral protease (Zawaski et al., 1993), and several matrix metalloproteases (Hu et al., 1994; Mackay et al., 1992). As discussed in Section 2.5.8, the activities of such proteases have been shown to be elevated after exposure to radiation/chemical carcinogens and reversed to normal levels after treatment with BBI. These lines of evidence have suggested that *c-fos* gene expression may be a critical target for both malignant transformation and the protease inhibitor suppression of malignant transformation.

4. IS BOWMAN-BIRK INHIBITOR TAKING THE PLACE OF α-ANTICHYMOTRYPSIN AS A DEFENSE ENZYME?

The effects of the anticarcinogenic protease inhibitors described in Section 2.5 are very unusual. Clearly BBI is an extremely potent protease inhibitor; BBI recently has been shown to have reaction properties better than any known physiologic protease inhibitor (Ware et al., 1997). While the ability of BBI to serve as a direct and potent protease inhibitor is unquestionable, it is possible that the effects of BBI on some of the levels of proteolytic activities serving as IME in cancer prevention studies may not all be direct effects, based on the catalytic activity of BBI.

Elevated levels of certain types of proteolytic activities are observed in radiation and chemical carcinogen-treated animals *in vivo* and *in vitro*, as described in Section 2.5.8.2. The ability of BBI to reduce these elevated levels of proteolytic activities in carcinogen-treated tissue *in vivo* and in cells *in vitro* is thought to be related to its ability to prevent carcinogenesis. Some of the data have suggested, however, that BBI is affecting the levels of proteolytic activities serving as IME in cancer prevention studies by altering the levels of expression of the genes coding for these proteolytic activities in cells, as described elsewhere (Kennedy and Manzone, 1995; Kennedy, 1995b). For these IME, it is known from various *in vivo* and *in vitro* studies that the BBI-produced changes in the levels of the IME never go below the endogenous levels observed in normal cells of tissues of the types being studied, suggesting that it is not simply the effect of a protease/protease inhibitor interaction that has been studied (i.e., with changes in the levels of the IME in animals and humans determined by substrate hydrolysis experiments). BBI clearly has such an indirect effect on at least one well-characterized protease, plasminogen activator (PA). It is known that BBI can prevent the induction of PA (Long et al., 1981; unpublished data), but has no effect on the catalytic activity of the enzyme, suggesting that it is involved in the regulation of PA. It is conceivable that BBI and other anticarcinogenic protease inhibitors are regulating the expression of protease genes in the same manner as previously observed for *c-myc* and *c-fos*, etc., as described in Section 2.5.8.1.

It is the author's hypothesis that BBI is capable of taking the place of an important regulatory enzyme in the body, α_1-antichymotrypsin, in certain, perhaps many, reactions. The chymotrypsin inhibitory activity of BBI (reviewed by Birk, 1974, 1976, 1985) is very similar to that of α_1-antichymotrypsin. When a person is wounded, α_1-antichymotrypsin is a major enzyme induced as an acute phase protein; the plasma concentration of this protease inhibitor increases rapidly and dramatically after a variety of traumatic events (reviewed by Travis and Salvesen, 1983). It is thought that α_1-antichymotrypsin is the most important physiologic protease inhibitor in the initial stages of the inflammatory process (Travis et al., 1978). When a plant is wounded, BBI or BBI-like molecules (BBI in soybeans and BBI-like protease inhibitors in other vegetables) are the major enzymes induced; these enzymes are thought to play an important role in plant defense mechanisms (e.g., Green and Ryan, 1972; Lee et al., 1986; Brown and Ryan, 1984). It is not inconceivable that α_1-antichymotrypsin and BBI play similar roles in the systems in which they operate. In fact, BBI and α_1-antichymotrypsin have several similar effects. Both BBI (Kennedy, 1993a; von Hofe et al., 1991; J. Ware, P. N. Newberne and A. R. Kennedy, unpublished data) and α_1-antichymotrypsin (reviewed by Travis and Salvesen, 1983) are highly anti-inflammatory. They both prevent the induction of active oxygen species in stimulated PMN (Frenkel et al., 1987; Kilpatrick et al., 1991), and they both directly inhibit the proteolytic activity of a major protease

involved in inflammation (cathepsin G) (Beatty et al., 1980; Travis et al., 1978; Laine et al., 1982; Larionova et al., 1994a). These effects would be expected to contribute to the anti-inflammatory activity of these compounds. The BBI's similarities to α_1-antichymotrypsin may explain its apparent ability to regulate numerous different processes, as α_1-antichymotrypsin is thought to play a major regulatory role in several different important metabolic processes. The rapid increase in production of α_1-antichymotrypsin in the acute phase is thought to be ensuring tight control over proteolytic activity in order to maintain homeostasis, and it is believed that α_1-antichymotrypsin is likely to play a far more important physiologic role than any of the other plasma protease inhibitors that have been studied (Travis et al., 1978). One of the mechanisms for control by α_1-antichymotrypsin is through the direct inhibition of proteases known to be involved in the activation of other proteases. As one example, it is known that mast cell chymase activates human interstitial procollagenase (Saarinen et al., 1994) and that α_1-antichymotrypsin inhibits mast cell chymase (Schechter et al., 1989). It will be difficult to demonstrate that BBI can take the place of α_1-antichymotrypsin, however, as animals without this enzyme do not exist; this is not surprising given the hypothesis that α_1-antichymotrypsin is an enzyme playing the major role in the defense of an organism.

It is conceivable that BBI will be most useful in conditions in which the natural defense mechanisms have failed; for example, in the treatment of inflammatory diseases such as IBD. It is known that α_1-antichymotrypsin levels are elevated in patients with IBD (Weeke and Jarnum, 1971; Travis and Salvesen, 1983). It is hypothesized that these elevated levels of α_1-antichymotrypsin are not quite high enough to control the disease in these patients and that a higher "dose" of chymotrypsin inhibitory activity, such as that which could be provided by BBI, would be enough to control the disease.

The significance of BBI studies may go far beyond its use as an anticarcinogenic agent. Its role as a powerful gene regulator could affect many diseases. For example, as described above, while the anticarcinogenic protease inhibitors, such as antipain and BBI, cannot affect the catalytic activity of PA, they can regulate PA gene expression (Long et al., 1981; Kennedy A. R., unpublished data). PA is a protease known to play a role in heart disease, diabetes, and several other diseases. It is known that soy protein dietary additions reduce heart disease, as measured by many different parameters (Messina, 1995). The traditional Japanese diet contains an exceptionally high level of soy products, as has been discussed elsewhere (Sipos, 1990; Doell et al., 1981). It is known that the Japanese are the longest-lived population in the industrialized world. (Metropolitan Insurance Companies, 1992) and have markedly reduced rates of several diseases common in the Western world, which include the common Western cancers (reviewed in Kennedy et al., 1993b). It has been observed that a BBI dietary addition leads to a significantly increased life span in mice (Kennedy

et al., 1993b). It is likely that BBI is affecting many processes yet to be studied. It plays a powerful regulatory role in many different systems and serves to bring various endpoints studied to the "right" or normal level observed in the system. For example, while BBI normally does not affect the levels of proteolytic activity in vivo, it brings carcinogen-elevated levels of activity to the normal levels usually observed (Messadi et al., 1986). Similarly, BBI reverses radiation/carcinogen elevated levels of c-myc expression both in vitro and in vivo (Chang et al., 1985, 1990; Chang and Kennedy, 1988, 1993; St. Clair et al., 1990b; St. Clair and St. Clair, 1991), gene amplification (Flick and Kennedy, 1991), etc. Thus, BBI serves to maintain many biological phenomena in a "normal" state. Protease inhibitors may play many different physiological roles in an organism; homology searches have revealed that several different heat shock proteins belong to the serine protease inhibitor superfamily (e.g., Takechi et al., 1992).

There is some evidence to suggest that protease inhibitors such as BBI are involved in protection at times of trauma in mammalian organisms. As an example, some protease inhibitors offer protection against radiation-induced lethality in vivo, as measured by survival levels in chickens and mice. The spectrum of protease inhibitors offering protection against radiation-induced mortality has suggested that a kallikrein-like enzyme, playing a role in vascular permeability, is likely to be involved in this effect (reviewed by Palladino et al., 1982). Similarly, in studies performed by Ware et al. (unpublished data), the administration of BBI in the diet significantly enhanced the survival levels of mice exposed to dextran sulfate sodium.

BBI administration has been shown to be beneficial for septic shock treatment. Jochum et al. (1981) have reported that when experimental endotoxemia was produced in dogs, the degradation of various plasma factors was significantly reduced when BBI was administered prior to gram-negative endotoxin exposure. Further studies by this group of researchers showed that BBI administered to dogs clearly reduced the endotoxin-induced decline in the plasma levels of the following clotting factors that were measured: antithrombin III, prothrombin, factor XIII, plasminogen, and α_2-antiplasmin, as well as complement factor C3 (Witte, 1979; Jochum et al., 1984). The studies presented by Jochum et al. (1984) indicate that treatment with BBI could be highly beneficial in generalized inflammatory processes like septicemia or septic shock. From their studies, they assume that BBI has the ability to prevent the nonspecific degradation of plasma factors and, therefore, contributes to maintaining the physiological balance of the organism.

5. POTENTIAL ADVERSE EFFECTS OF ANTICARCINOGENIC PROTEASE INHIBITORS
5.1. Effects of Protease Inhibitors on Growth

In the past, protease inhibitors in vegetables have been regarded as anti-nutritional substances, primarily because growth-inhibitory effects have been attributed to them.

Questions about the role of protease inhibitors in nutrition began with the findings of Osborne and Mendel (1917) that soybeans would not support the growth of young rats unless they were heat treated. As it was thought that protease inhibitors were inactivated by heat treatment, it was assumed that it was the inactivation of protease inhibitors by the heat treatment that led to soybean products that did not suppress growth. In fact, the Bowman-Birk type of protease inhibitors are quite resistant to heat treatment and are found in an active form in many processed foods, such as canned chick peas, kidney beans, etc. (Yavelow *et al.*, 1982). The evidence suggesting that protease inhibitors are anti-nutritional has been largely circumstantial, based on studies that have not utilized purified compounds. When purified protease inhibitors have been studied for their effects on growth, the studies have clearly shown that protease inhibitors do not have the deleterious effects on growth that have been widely attributed to them, as discussed elsewhere (Birk, 1993).

While the protease inhibitors in soybeans do not appear to have the primary suppressive effects on animal growth, it is clear that some components of soybeans do have growth-suppressive effects. There are many anti-nutritional and toxic factors in legumes, as has been reviewed by Liener (1989). For example, soybeans contain high levels of tannins (polyphenolic substances). Tannins are known to inhibit digestive enzymes in a nonspecific manner (Gallaher and Schneeman, 1986). Tannins are responsible for poor digestibility of proteins and growth inhibition when fed to animals; the anti-nutritional characteristics of tannins have been discussed in detail elsewhere (Liener, 1989; Rackis *et al.*, 1986). Other anti-nutritional substances found in soybeans (and other legumes) include hemagglutinins (lectins), goitrogens, cyanogens, lathyrogens, phytate (phytic acid), flatulence producers, and gossypol (Liener, 1989). While any or all of these substances could contribute to the growth-suppressive effects of raw soybeans, it is likely that the soybean protein itself is the major factor producing growth suppression (Gallaher and Schneeman, 1986). When trypsin inhibitors were removed from raw soybean meal by affinity chromatography and the raw meal lacking protease inhibitors was fed to rats, the degree of growth depression was only a bit less than the raw meal containing the trypsin inhibitors (Kakade *et al.*, 1973). This experiment suggested that much of the growth-suppressive effect was due to the protein itself. In their native state, plant proteins are refractory to proteolytic attack, which results in poor amino acid availability (Grau and Carroll, 1958; Bozzini and Silano, 1978). As heat treatment of soybean meal is known to increase the digestibility of soybean protein, heat treatment thus would bring about an alleviation of growth depression produced by soybean protein in its native state (Gallaher and Schneeman, 1986). While the precise contribution of protease inhibitors to the growth-suppressing effects of raw soybeans is controversial, it is clear that heat treatment of soybeans results in products that support the normal growth of animals.

5.2. Effects of Protease Inhibitors on the Pancreas

5.2.1. Regulation of pancreatic secretions/effects of protease inhibitors on pancreatic secretions.

For the rat, a negative feedback mechanism exists for pancreatic enzyme secretion, and cholecystokinin (CCK) is known to be a major regulatory hormone (Green and Lyman, 1972). CCK is secreted by cells of the proximal small intestine as a response to an increase in the level of trypsin activity in the intestinal lumen (Green and Lyman, 1972). CCK-pancreozymin is the humoral agent released from the intestine; it has the ability to stimulate pancreatic secretion and cause both pancreatic hypertrophy and hyperplasia (Rothman and Wells, 1967; Mainz *et al.*, 1973; Yanatori and Fujita, 1976). The exact details of the feedback response are still controversial. Evidence has been presented that a "monitor peptide" is secreted with the pancreatic juice and signals CCK secretion by the intestinal mucosa (Fushiki and Iwai, 1989; Miyasaka *et al.*, 1989). If the intestinal mucosa is low in trypsin inhibitor activity, these peptides are destroyed by proteolytic activity and CCK secretion is reduced. CCK secretion is increased when trypsin inhibitors are present in the diet; it is assumed that trypsin inhibition protects the CCK-releasing peptides from being destroyed. It is thought that CCK induces hyperplasia and hypertrophy of the acinar tissue (as well as having some effect on the ductal elements) of the pancreas as a response to greater needs for pancreatic functions (Morgan, 1987). CCK is thought to be an essential promoter involved in pancreatic carcinogenesis in the rat (Roebuck and Longnecker, 1993).

Whether a feedback system involving CCK exists in humans, or in fact, in any species other than rats, is not known, as discussed elsewhere (Roebuck and Longnecker, 1993). It is known that the administration of camostate, known to induce CCK in rats (Roebuck and Longnecker, 1993), does not induce an increase in plasma levels of CCK in humans (Watanabe *et al.*, 1986; Adler *et al.*, 1986, 1988). In fact, there is evidence that dietary soybean protease inhibitors lead to a reduction in plasma levels of CCK in humans (Lu *et al.*, 1995). Although controversial, there is some evidence that humans do have feedback control of pancreatic enzyme secretions, as discussed by Toskes (1986) and Liener *et al.* (1988). If such a system does exist for the human pancreas, which could conceivably lead to increased plasma levels of CCK, this does not mean that the human pancreas will respond to SBTIs with enlargement or carcinogenic changes. A negative feedback mechanism for pancreatic enzyme secretion (similar to that of rats) exists in hamsters (Andrén-Sandberg and Ihse, 1983), pigs, and calves, but these species do not develop pancreatic cancer (or even enlargement in the case of pigs and calves) in response to trypsin inhibitors (reviewed by Gallaher and Schneeman, 1986). The existence of feedback control of the pancreas appears to be absent in some species, such as the dog (Sale *et al.*, 1977; Diaz *et al.*, 1982).

The effect of protease inhibitors on the negative feedback control system is not specific; protease inhibitors affect the system in the same manner as do other proteins,

but are a somewhat more potent stimulus (Green and Lyman, 1972; Green et al., 1973). It is of interest that proteins have the same "deleterious" effects on the rat pancreas attributed to protease inhibitors. Raw soybean protein fractions lacking trypsin inhibitor activity cause pancreatic enlargement in rats in the same manner as do the protease inhibitor-containing fractions (Naim et al., 1982).

It is specifically trypsin inhibition, and not chymotrypsin inhibition, that is involved in triggering the feedback response, and ultimately the production of pancreatic enlargement in rats, as reviewed by Birk (1993). As it is chymotrypsin inhibition that is involved in the anticarcinogenic activity of BBI (Kennedy, 1985a; Yavelow et al., 1985), the anticarcinogenic activity can be separated from the TI activity, which presumably has the potential to produce deleterious side effects on the pancreas in some species.

5.2.2. Histopathological changes in the pancreas associated with soybean products.

High levels of unheated soybean products in the diet of rats have been associated with pancreatic hyperplasia and hypertrophy; in a few rats fed in this manner for very long periods of time, pancreatic cancer developed (Naim et al., 1982; McGuiness et al., 1980; Gumbmann et al., 1985; Crass and Morgan, 1982; as reviewed by McGuiness et al., 1984; Morgan, 1987; Morgan et al., 1977). Although the carcinogenic effect of soybeans on the rat pancreas has been widely attributed to the protease inhibitors in soybeans (Liener and Kakade, 1980; Rackis et al., 1986; Flavin, 1982), the evidence for this is not clear.

There appears to be no doubt that the soybean protease inhibitors are involved in the stimulation of growth in the rat pancreas, causing primarily hypertrophy and some hyperplasia (Melmed et al., 1976; Crass and Morgan, 1982; Kakade et al., 1967), but whether these lesions are related to the development of pancreatic cancer is controversial (Birk, 1993). As there are some species (e.g., mice) that respond to soybean protease inhibitors by developing pancreatic hyperplasia and hypertrophy, but do not develop cancer, and there are other species (e.g., hamsters) that respond to soybean protease inhibitors with pancreatic enlargement and a reduced pancreatic cancer risk (Liener and Hasdai, 1986), these phenomena may not be related. Even in rats fed soybean protease inhibitors, the occurrence of hyperplasia/hypertrophy and cancer in the pancreas are separable phenomena (Richter and Schneeman, 1987). Other components of soybeans could be responsible for the carcinogenic effect, as discussed in Sections 5.2.3, 5.2.4, and 5.2.7.

Enlargement of the pancreas associated with soybean protease inhibitors is a readily reversible process (McGuiness et al., 1984), as expected for the effects of a promoting agent (reviewed by Weinstein, 1978). (The evidence for considering soybean products as "promoters" of rat pancreatic carcinogenesis is discussed in Section 5.2.4.) Even when rats are fed on raw soya flour diets for as long as 6 months and then receive non-soya-containing diets, the pancreatic weights, protein contents, and morphological appearances

of the pancreata revert to normal in a very short period of time (McGuiness et al., 1984).

The pancreata of most species do not respond to high levels of soybeans in the diet, as does the rat pancreas (Birk, 1974, 1976, 1985, 1993; Folsch et al., 1974). Examples of species whose pancreata do not respond to soybean products in the manner observed for rats are as follows: dogs (Wolf and Cowan, 1975; Patten et al., 1971a,b), the adult guinea pig (Patten et al., 1973), calves (Kakade et al., 1976; Gorrill and Thomas, 1967), pigs (Struthers et al., 1983; Hooks et al., 1965; Yen et al., 1977), cebus monkeys (Ausman et al., 1985; Struthers et al., 1983), and chacma baboons (Robbins et al., 1988).

There are a few species whose pancreata respond with enlargement, as do the pancreata of rats, to high levels of soybeans in the diet. These species include chickens (Chernick et al., 1948; Nitsan and Alumot, 1964; Gertler and Nitsan, 1970; Nitsan and Nir, 1977), the young, but not adult, guinea pig (Patten et al., 1973), quails (Birk, 1985; Madar et al., 1974), mice (Schingoethe et al., 1970), and hamsters (Hasdai and Liener, 1983). From the data concerning the species variation in response, it has been suggested (Kakade et al., 1976; Liener, 1979a,b; Liener and Kakade, 1980) that animals whose pancreata are 0.3% of body weight or more (such as the mouse, chick, rat, and young guinea pig) show pancreatic enlargement with feeding of soybean trypsin inhibitors, while those animals whose pancreata are less than 0.3% of body weight (the adult guinea pig, pig, dog, and calf) do not exhibit pancreatic enlargement. With this relationship, the human pancreas is not expected to enlarge due to trypsin inhibitors, as it weights about 0.09–0.12% of body weight.

Thus, the available evidence suggests that soybean protease inhibitors will not bring about pancreatic enlargement. Even if they do, however, this does not mean that these protease inhibitors will produce pancreatic cancer. As noted above, pancreatic enlargement produced by soybean protease inhibitors does not lead to pancreatic cancer in mice, and in hamsters, the soybean protease inhibitors suppress pancreatic cancer development, even though they cause pancreatic enlargement (Liener and Hasdai, 1986).

5.2.3. Is soybean product-associated pancreatic cancer in rats due to protease inhibitors?

As has been discussed elsewhere (Roebuck and Longnecker, 1993), when rats are fed a diet containing raw, full-fat soybean flour, pancreatic cancer can occur and carcinogen-induced pancreatic carcinogenesis can be enhanced, but whether the effect is due to protease inhibitors is not clear. Several studies have suggested that protease inhibitors may be only partially responsible for the deleterious effects of soybeans on the pancreas. For example, Kakade et al. (1973) and Liener (1979a) present data showing that trypsin inhibitors are responsible for only approximately 40% of the stimulatory effects of raw soy flour on the pancreas. The true effect may be even less than that estimate. It is known that there is a high content

of unsaturated fat in soybeans [raw soy flour contains approximately 20% fat (Richter and Schneeman, 1987)]; the unsaturated fat from soybeans is known to enhance pancreatic carcinogenesis in rats (Roebuck et al., 1987). When soybeans are defatted, the hyperplastic, hypertrophic, and carcinogenic effects of the soybean products (containing high levels of the soybean protease inhibitors) in the pancreas of rats are not observed (for a review, see Richter and Schneeman, 1987). Further evidence that the soybeans protease inhibitors may not be responsible for the deleterious effects of raw soybeans on the pancreas is that protein fractions lacking TI activity cause pancreatic enlargement in rats (Naim et al., 1982).

The fact that the hypertrophic, hyperplastic, and carcinogenic effects of raw soybean products on the pancreas can be abolished by heat treatment of the soybean flour (McGuiness et al., 1980; Folsch et al., 1974; Roebuck et al., 1987) suggests that BBI (and the other BBI-type protease inhibitors in soybeans) is not responsible for these effects, as this protease inhibitor is highly resistant to degradation by normal levels of heat treatment[†] (Bowman, 1946; Birk, 1961).

5.2.4. Relationship of experiments on azaserine-induced pancreatic cancer to soybean-associated pancreatic pathology in rats.

In the experiments that have been performed to determine the effects of constituents of soybeans on the pancreas, pure compounds have not been used, so that observed effects often can be attributed to other components of the soybeans or the diet utilized. In several experiments performed on azaserine-induced rat pancreatic carcinogenesis, a synthetic protease inhibitor called FOY-305 (or camostate) has been used, as discussed by Roebuck and Longnecker (1993). These studies suggest that this protease inhibitor might have two opposing effects on pancreatic carcinogenesis: a promoting effect on early preneoplastic lesions, caused by its ability to enhance CCK secretion, but then a direct suppressive effect on pancreatic carcinogenesis after its absorption from the intestine and transport to the pancreas. It has been shown that growth of basophilic putative neoplastic foci in the pancreas is inhibited by camostate (and stimulated by CCK) (Douglas et al., 1989). As suggested (Roebuck and Longnecker, 1993), these studies on the pancreas suggest that the overall effect of protease inhibitors absorbed from the GI tract may be to suppress pancreatic carcinogenesis, even in the rat model system. In azaserine-induced pancreatic carcinogenesis in rats, protease inhibitors appear to act as promoters only during the early postinitiation phase (i.e., the first 2 months and not the last 2 months of the postinitiation phase) of carcinogenesis (Roebuck et al., 1987). In these studies, both a protease inhibitor-containing protein fraction and a fraction containing unsaturated fat from soybeans increased the size of azaserine-induced preneoplastic foci in the pancreas, but did not increase the number of foci occurring in the "initiated" pancreas (Roebuck et al., 1987). These studies suggest that the soybean protease inhibitor-containing fraction (as well as the fraction containing unsaturated fat) can act as a growth promoter for carcinogen-induced pancreatic carcinogenesis in rats, but the protease inhibitor-containing fraction had no ability to induce cancer by itself or to act as a co-carcinogen with azaserine to enhance pancreatic carcinogenesis. A purified trypsin inhibitor has also been shown to act as a promoter for azaserine-induced pancreatic carcinogenesis (Douglas et al., 1989). Similar conclusions have been reached in experiments involving spontaneous pancreatic carcinogenesis; the data suggest that soybean products do not cause cancer by themselves, but instead, can serve as a promoter for multistage carcinogenesis in the pancreas (McGuiness et al., 1984).

In the studies that have shown pancreatic cancer development in rats maintained on high levels of soybeans in the diet, "spontaneous" carcinogenesis could be induced by a variety of carcinogens in the environment of the animals. As an example, N-nitrosamines, which are known to cause pancreatic cancer in animals (McGuiness et al., 1984), have been found in animal food and bedding material (Silverman and Adams, 1983).

5.2.5. Apparent dose-response relationship for pancreatic pathology in rats.

Pancreatic pathology from soybean products has only been observed at very high levels in the diet. In these studies, lower levels have not led to these adverse effects; levels of protease inhibitors of approximately 4% of the diet or more appear to be necessary to observe statistically significant results (e.g., Myers et al., 1991; Gumbmann et al., 1985), as reviewed elsewhere (Kennedy, 1995a). At levels of protease inhibitors in the diet that are appropriate for cancer prevention (approximately 0.1% or less, as described in Section 6), pancreatic pathology in rats is not observed. For example, in the USDA Trypsin Inhibitor Study (Gumbmann et al., 1985), the results for pancreatic adenoma incidence (number of rats with adenomas/number of rats studied), with TI activity at 0.1% of the diet (93 mg TI/100 g diet), are 0/107 vs. the control incidence of 1/104.

There is thought to be a threshold in rats for both stimulation of CCK and for bringing about pancreatic enlargement. The threshold for negative feedback regulation by luminal proteases occurs when luminal protease activity is reduced by 90% in the rat; at this point, increased pancreatic secretion occurs (Miyasaka and Green, 1984). It is widely believed that there is also a threshold for pancreatic pathology (hypertrophy) in rats (Rackis et al., 1975; Churella et al., 1976; McGuiness et al., 1984; Liener, 1989).

[†]Whereas the proteolytic activity of BBI is relatively unaffected by heat treatment, such treatment does destroy most of the protease inhibitor activity in soybeans. Heat treatment not only inactivates most of the trypsin inhibitors, but also destroys some of the amino acids and generally reduces amino acid availability. As the amount of heat necessary to destroy all of the trypsin inhibitor activity would damage the nutritive value of proteins, most commercially available soy products are heat treated to an extent that results in 5–20% of the original TI activity remaining intact (Rackis et al., 1986).

This threshold for pancreatic enlargement in rats occurs when the animals are fed raw soy flour in an amount that represents 5% of the total protein in the diet (McGuiness et al., 1984).

5.2.6. Acinar vs. ductal origin of pancreatic tumors.
Rats develop pancreatic pathology of acinar cell origin from azaserine and soybean products. Acinar cell neoplasms are rarely seen in the human pancreas; this type of pancreatic cancer is observed in only approximately 4% of human pancreatic cancer (Pour, 1991). The common type of human pancreatic cancer is of ductal/ductular origin; ductal neoplasms account for over 90% of human pancreatic cancer (Pour, 1991). Nitrosamine-induced pancreatic carcinogenesis in hamsters is the experimental model system that results in pancreatic neoplasms of ductal origin. Many modifying agents for carcinogenesis have opposite effects on the two types of pancreatic cancer development (i.e., acinar cell pancreatic neoplasms in rats and neoplasms of ductal origin in hamsters), as has been reviewed (Pour, 1991). One example of such opposing effects involves soy flour treatment. Raw soy flour dietary additions containing a high level of soy TI activity lead to highly significant reductions in the levels of experimentally induced pancreatic carcinogenesis in hamsters (Hasdai and Liener, 1983; Liener and Hasdai, 1986; Permert et al., 1993; Pour, 1991). This soybean flour-suppressing effect on pancreatic tumors of ductal origin suggests that high levels of soybean products in the diet are likely to lead to a reduction in human pancreatic cancer. This, in fact, is what has been observed in epidemiologic studies, as described in Section 5.2.8.2.

5.2.7. The role of fat in rat pancreatic pathology.
In studies claiming to demonstrate that soybean products are associated with pancreatic pathology in rats, the soybean fat has been studied along with the soybean protease inhibitors. Thus, conclusions about the effects of protease inhibitors in the rat model system are problematic. There are only a few studies (Gumbmann et al., 1985, 1989; Spangler et al., 1985) in which the soybean products studied have been defatted. (Although the soybean products used were defatted, corn oil was used in the studies; this is problematic for reasons described below.) In these studies in which defatted soybean protease inhibitor preparations were utilized, there were no significant differences in the incidence of pancreatic cancer—only acinar adenomas were observed in rats and not even adenomas (or other pathologic alterations) were observed in mice. In these studies, the fact that pancreatic hyperplasia and hypertrophy are separable from adenoma development is clear (given the differences observed between the rat and mouse studies), and there is no evidence suggesting that the adenomas occurring in rats lead to cancer. These studies show very clearly that when soybeans are defatted, pancreatic cancer does not occur in rats, and suggest that the soybean fat present in previous studies (e.g., McGuiness et al., 1984) played a very important role

in the development of pancreatic cancer in rats fed high levels of full-fat soybean flour for long periods of time.

Other types of fat are also important in studies of rat pancreatic carcinogenesis. As one example, high levels of corn oil (7.88%) were added as the dietary fat in the USDA TI study (Gumbmann et al., 1985; Spangler et al., 1985). It is known that considerably lower levels of corn oil than were utilized in that study have been shown to lead to highly significant increases in the levels of pancreatic hyperplasia and acinar adenomas in both male and female rats (Boorman and Eustis, 1984; Boorman et al., 1987; Haseman et al., 1985; National Toxicology Program, 1994). Thus, the use of corn oil in studies of rat pancreatic carcinogenesis greatly complicates the analysis of the contribution of soybean protease inhibitors to the development of pancreatic pathology.

5.2.8. Pancreatic cancer in humans/relationship of soybean products to pancreatic cancer.
5.2.8.1. *Soybean products are unlikely to cause adverse effects in the human pancreas*. While the causes of human pancreatic cancer are unknown, there is a strong association with cigarette smoking (Mack, 1982), as well as an association with high levels of dietary fat (Norell et al., 1986; Durbec et al., 1983; Carroll and Khor, 1975; Wynder, 1975; Gold et al., 1985). Experimental pancreatic carcinogenesis in rats has also shown a strong association with high levels of dietary fat, as discussed in Section 5.2.7.

There are several reasons to believe that soybean-derived protease inhibitors and other soybean constituents will not have a carcinogenic effect on the human pancreas as they may have on the rat pancreas; they are as follows:

(1) Human trypsin is more resistant to inhibition by the soybean-derived protease inhibitors than is the trypsin of other mammals; it is expected that dietary soybean-derived trypsin inhibitors will not have a sufficiently strong effect to bring about human pancreatic pathology (reviewed by Flavin, 1982).

(2) The size (percentage of body weight) of the human pancreas is such that it falls into a classification of species that do not respond to soybean trypsin inhibitors (products) by pancreas enlargement (or cancer development), as discussed in detail in Section 5.2.2.

(3) Rats have different essential amino acid requirements than do humans; of particular importance is that their requirements for sulfur-containing amino acids, such as methionine, are not like those of humans (Bodwell and Hopkins, 1985; Ford, 1981). The metabolic pathway by which rats (chicks and presumably other species responding to soybean-derived trypsin inhibitors with pancreatic enlargement) metabolize soybean products is unusual and clearly different from the pathways operating in humans (for example, see Young et al., 1984; Scrimshaw et al., 1983). Since soybean protein is known for its deficiency in sulfur-containing amino acids (Liener and Kakade, 1980), rats respond to this food source in a manner unlike that of most species, in-

cluding humans. Rats are considered a poor model for humans in the determination of the soybean protein efficiency ratio, an assay utilized to assess the nutritional qualities of human food proteins; alternatives to the rat model system are discussed in detail elsewhere (for examples, see Bodwell and Hopkins, 1985; Torun et al., 1982; Pineda et al., 1982; Soy Protein Council, 1987). Thus, rats are considered an unsuitable species for evaluation of effects of soybean protease inhibitors on any organ system, including the pancreas, and the results of studies on soybean products in rats cannot be assumed to represent the likely response of humans to those products.

(4) Even if there were a clear dose-response relationship between TI intake and incidence of certain pancreatic abnormalities in the rat pancreas, this is not convincing evidence that protease inhibitors cause pancreatic cancer. As discussed elsewhere (Kennedy, 1993a), protease inhibitors are clearly associated with increased growth in the rat pancreas, which leads to hypertrophy and hyperplasia, but it is not at all clear whether hypertrophy and hyperplasia are related to cancer development in the pancreas. There are species that have these pancreatic lesions and do not get cancer and other species that have reduced cancer rates in the pancreas, even though they have pancreatic hypertrophy and hyperplasia. Thus, hypertrophy and hyperplasia are clearly separable phenomena from the development of cancer in the pancreas, as discussed in more detail elsewhere (Birk, 1993; Kennedy, 1993a, 1996a).

5.2.8.2. Soybean products may cause a decrease in the rates of human pancreatic cancer. Although the effect of soybean-derived protease inhibitors on human pancreatic carcinogenesis is unknown, there are normal human populations with high levels of protease inhibitors in the diet (Sipos, 1990; Doell et al., 1981) that show no increased risk of pancreatic cancer [e.g., the Japanese and Seventh-Day Adventists (Armstrong and Doll, 1975; Phillips, 1975; Phillips et al., 1980)]. The Seventh-Day Adventists are known to have high dietary levels of protease inhibitor activity derived from soybeans and other legumes (primarily peas and lentils). Studies designed specifically to determine pancreatic cancer rates in this human population have clearly shown reduced rates of pancreatic cancer (Mills et al., 1988).

5.2.8.3. Summary of data on pancreatic effects from soybean protease inhibitors. As described in detail in Section 5.2, the interpretation of the results from studies utilizing the development of acinar cell neoplasms in the rat model system for evaluation of effects on the pancreas from soybean products is problematic due to a number of different factors, including the amount and type of fat present, etc. The most important question to be asked about the results of the rat pancreatic carcinogenesis studies, however, is whether such studies performed in a system in which acinar adenocarcinomas develop have any relevance to human pancreatic

carcinogenesis. It is known that almost all human pancreatic cancer is of ductal origin and that soybean products containing high levels of the soybean protease inhibitors have major suppressive effects on pancreatic cancer of ductal origin in animals (reviewed by Pour, 1991). These results from animal experiments are likely to reflect the response of the human pancreas to soybean-derived protease inhibitors. The available evidence suggests that dietary soybean-derived protease inhibitors are likely to lead to a reduction in the incidence and mortality rates of human pancreatic cancer. This is the opposite result from that expected on the basis of the animal studies performed in rats. Other phenomena thought to be related to pancreatic cancer development also give opposite responses in the pancreata of rats compared with those of humans. For example, high levels of dietary soybean protease inhibitors lead to increased plasma levels of CCK in rats (Liddle et al., 1984; Adrian et al., 1982), but decreased plasma levels of CCK in humans (Lu et al., 1995).

5.3. Potential Effects of Protease Inhibitors on the Immune System

Another potential toxicity problem for cancer prevention by protease inhibitors involves a possible effect of protease inhibitors on the immune system. This is considered a potential toxicity problem due to the report by Goldstein et al. (1979), which showed that protease inhibitors can prevent the production of the superoxide anion radical and H_2O_2 in PMN stimulated by tumor promoting agents. Even though this effect of protease inhibitors is specifically in response to an undesirable cellular effect brought about by tumor promoting agents, the results reported by Goldstein et al. (1979) have suggested to some that protease inhibitors might interfere with the normal functioning of the immune system.

In addition, the possible role of proteases in the normal functioning of the immune system (in particular, neutrophils and macrophages) has also raised the question of whether or not protease inhibitors in some way might compromise the normal functioning of the immune system. For several reasons, an adverse effect of dietary protease inhibitors on the immune system is unlikely. There are currently two major hypotheses on the mechanism of target-cell killing by macrophages: (1) cytolysis dependent on reactive oxygen intermediates and (2) cytolysis dependent on lysosomal enzymes (Leb et al., 1985). α-Tocopherol was found to be highly effective at inhibiting both antibody-dependent monocyte cytotoxicity and phorbol-myristate-acetate (PMA)-induced monocyte cytotoxicity (Corwin and Gordon, 1982). It generally is thought that this indicates that the production of H_2O_2 and oxygen free radicals are of primary importance for macrophage function. In spite of this, α-tocopherol is commonly used as a dietary supplement with no apparent ill effects. In fact, a slight stimulatory effect of α-tocopherol on the immune system (i.e., the opposite effect compared with that observed *in vitro*) has been

observed *in vivo* (Corwin and Gordon, 1982; Lim et al., 1981). Two protease inhibitors, an ovomucoid trypsin inhibitor and SBTI, have been studied in separate investigations for their inhibitory effect on cell killing (Leb et al., 1985). In the second, SBTI was tested for inhibition of elastolytic activity and found to be negative (Chapman and Stone, 1984). With respect to neutrophils, proteolytic activity is thought to play a major role in unwanted tissue destruction, such as in emphysema. Consequently, there has been much medical research to find a protease inhibitor that might antagonize this protease-associated pathological condition. While SBTI was found to be more effective at inhibiting the elastolytic activity of neutrophils than macrophages *in vitro*, it is not expected to be effective *in vivo* (Harlan et al., 1981). The close association between effector cells and target cells makes access of protease inhibitors to proteases involved in tissue destruction or cell killing difficult (this would be true for neutrophils, as well as for macrophages). Thus, it is highly unlikely that there will be deleterious effects of protease inhibitors on the normal functioning of the immune system. Thus far, studies performed to determine whether BBI has an effect on cells of the immune system or the normal functioning of the immune system have shown no adverse effects (Goldfarb et al., 1989; Maki and Kennedy, 1992; Maki et al., 1994).

6. AMOUNTS OF BOWMAN-BIRK INHIBITOR/ SOY PRODUCTS NECESSARY FOR ANTICARCINOGENIC EFFECTS

Evidence has been presented that the amount of protease inhibitor activity from soybeans in the traditional Japanese diet could account for a decreased cancer risk, as discussed in detail elsewhere (Kennedy, 1993a,c; Kennedy et al., 1993b). The data suggest that even the amount of protease inhibitor activity in a single serving of tofu (1 cup) per day could have some protective effect against cancer development. Animal carcinogenesis studies have demonstrated that dietary levels as low as 0.01% BBI/BBIC can suppress liver carcinogenesis in mice (St. Clair et al., 1990a) and colon carcinogenesis in rats (A. R. Kennedy, unpublished data). (Lower dietary levels of BBI/BBIC could also be effective at suppressing cancer development, but have not been studied yet.) If it is assumed that the normal human dietary intake is 500 g of food, 0.01% of 500 g would be 50 mg of dietary protease inhibitor activity that would be necessary in the human diet for the prevention of some kinds of cancer. The protease inhibitor content of many foods is known (e.g., Rackis et al., 1986; Liener, 1979b; Liener and Kakade, 1980; Doell et al., 1981; Billings et al., 1990b). For example, it has been determined that tofu contains approximately 150 mg/cup of protease inhibitor activity (Kennedy, 1993a). It is assumed that SBTI (also known as the Kunitz inhibitor, etc.) is removed from soybeans during their processing for the production of tofu (discussed by Liener, 1979b; and Liener and Kakade, 1980), leaving 5 other protease inhibitors, which are all members of the Bowman-Birk family of protease inhibitors (Hwang et al., 1977). Of these five different protease inhibitors present in tofu, only BBI itself contains CI activity (Hwang et al., 1977). As the amounts of these members of the BBI family of protease inhibitors in soybeans are approximately the same, it could be assumed that the CI activity of tofu would be approximately 30 mg/cup (i.e., one-fifth of 150 mg/cup). Although the epidemiologic data are somewhat inconsistent, there are approximately 20 studies suggesting that tofu intake reduces cancer risk, as has been reviewed elsewhere (Messina et al., 1994). In studies exhibiting a reduced cancer risk from tofu (e.g., Lee et al., 1991; Severson et al., 1989; Lee et al., 1995), the amount of tofu necessary for a protective effect against cancer is approximately 1 serving per day (compared with infrequent consumption) (M. Messina, personal communication). With the assumption that a serving of tofu is 1 cup, this amount of tofu would contain approximately 30 CI units. 30 CI units per day is expected to have a protective effect against the development of some forms of human cancer, if the results from animal studies are extrapolated to humans, as described above. This dose is comparable with the lowest amount of CI activity currently being studied in human/BBIC trials, 25 CI units/day. This amount of CI activity is expected to have a marginal/borderline effect on human carcinogenesis; considerably higher doses are thought to be necessary to achieve the maximum cancer preventive effect of BBIC, as discussed elsewhere (Kennedy et al., 1993b; Kennedy, 1993a).

7. SUMMARY

Protease inhibitors are highly promising as human cancer chemopreventive agents. For all cancer chemopreventive agents, potential toxicity problems need to be addressed. As discussed in detail in Section 5, certain adverse health effects have been attributed to protease inhibitors. It is perceived that the problems that could result from the chemoprevention of cancer with dietary protease inhibitors are (1) pathologic alterations could develop in the pancreas while cancer is being inhibited in other organs and (2) there may be decreased protein utilization, resulting in weight loss. As discussed in detail in this article, much of the evidence implicating protease inhibitors as causative agents for growth suppression in young animals and in rat pancreatic pathology/cancer development is circumstantial. It is now clear that it is not the soybean protease inhibitors causing growth suppression in young animals, as has been reviewed by Birk (1993). The assumption that the soybean protease inhibitors are causally related to the development of pancreatic cancer in rats may also be incorrect. While there is evidence that high dietary levels of soybean protease inhibitors cause pancreatic hyperplasia/hypertrophy in rats, there is much evidence to suggest that pancreatic hyperplasia/hypertrophy is not related to pancreatic cancer development, as discussed in detail in Section 5.2. As one example, results presented by Liener and Hasdai (1986) in-

dicate that pancreatic enlargement, presumably caused by hyperplasia/hypertrophy at the cellular level, can be associated with a decreased pancreatic cancer risk.

The rat pancreas is quite unusual in its response to soybean-derived protease inhibitors; the human pancreas is not expected to respond to soybean protease inhibitors with hyperplasia, hypertrophy, adenoma, or cancer development. Of particular relevance to the question of whether the use of high dietary levels of soybean products could lead to the development of human pancreatic cancer is the epidemiologic evidence. The populations having high levels of soybean products and protease inhibitor activity in their diets, such as the Japanese and the Seventh-Day Adventists (Sipos, 1990; Doell et al., 1981), do not have elevated risks of pancreatic cancer (as reviewed by Kennedy, 1993a; Fontham and Correa, 1993; Mills et al., 1988; Armstrong and Doll, 1975; Phillips, 1975; Phillips et al., 1980). In fact, some of these populations have significantly reduced rates of pancreatic cancer development (Mills et al., 1988). Thus, the available epidemiological evidence suggests that the human pancreas is likely to respond to soybean products with a reduced cancer rate. This would not be surprising, as 90% of human pancreatic cancer is ductal in origin (Pour, 1991) and pancreatic carcinogenesis of ductal origin in animals is reduced with soybean flour dietary additions (Hasdai and Liener, 1983; Liener and Hasdai, 1986; Permert et al., 1993; Pour, 1991). Whether there will be adverse or beneficial effects in the human pancreas associated with the administration of soybean protease inhibitors, or soybean products in general, will never be completely determined with epidemiologic correlations studies, however. Thus, it will be important to monitor the response of the human pancreas to the administration of soybean products as cancer preventive agents in human trials. It is believed that a suitable level of protease inhibitors in the diet can be found that will not lead to the problems cited above, since no undesirable side effects, including pancreatic changes, decreased growth rate or decline in general health, in animals maintained on high levels of anticarcinogenic dietary protease inhibitor activity for as long as their entire life span have been observed, as discussed in detail in this article. Thus, it is highly likely that protease inhibitor supplementation to the diet will prevent the development of cancer at several different sites and of many different types without adverse health effects in human populations.

References

Adler, G., Mullenhoff, A., Bozkurt, T., Koop, I., Goke, B., Beglinger, C. and Arnold, R. (1986) Pancreatic function and plasma CCK in humans after ingestion of a proteinase inhibitor (FOY-305). Dig. Dis. Sci. 31: 1123.

Adler, G., Mullenhoff, A. Bozkurt, T., Goke, B., Koop, I. and Arnold, R. (1988) Comparison of the effect of single and repeated administrations of a protease inhibitor (camostate) on pancreatic secretion in man. Scand. J. Gastroenterol. 23: 158–162.

Adrian, T. E., Pasquali, C., Pescosta, F., Bacarese-Hamilton, A. J. and Bloom, S. R. (1982) Soy-induced pancreatic hypertrophy and rise of circulating cholecystokinin. Gut 23: A889.

Andrén-Sandberg, A. and Ihse, I. (1983) Regulatory effects on the pancreas of intraduodenal pancreatic juice and trypsin in the Syrian golden hamster. Scand. J. Gastroenterol. 18: 697–706.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) Phorbol ester-inducible gene contains a common cis element recognized by a TPA-modulated trans-acting factor. Cell 49: 729–739.

Armstrong, B. and Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary factors. Int. J. Cancer 15: 617–631.

Ashendel, C. L. (1985) The phorbol ester receptor: a phospholipid-regulated protein kinase. Biochim. Biophys. Acta 822: 219–242.

Ausman, L. M., Harwood, J. P., King, N. W., Sehgal, P. K., Nicolosi, R. J., Hegsted, M. D., Liener, I. E., Donatucci, D. and Tarcza, J. (1985) The effects of long-term soy protein and milk protein feeding on the pancreas of Cebus albifrons monkeys. J. Nutr. 115: 1691–1701.

Baturay, N. Z. and Kennedy, A. R. (1986) Pyrene acts as a cocarcinogen with the carcinogens, benzo(a)pyrene, β-propiolactone and radiation in the induction of malignant transformation of cultured mouse fibroblasts; soybean extract containing the Bowman-Birk inhibitor acts as an anticarcinogen. Cell Biol. Toxicol. 2: 21–32.

Beatty, K., Bieth, J. and Travis, J. (1980) Kinetics of association of serine proteinases with native and oxidized α-1-proteinase inhibitor and α-1-antichymotrypsin. J. Biol. Chem. 255: 3931–3934.

Becker, F. F. (1981) Inhibition of spontaneous hepatocarcinogenesis in C3H/Hen mice by Edi Pro A, an isolated soy protein. Carcinogenesis 2: 1213–1214.

Berenblum, I., Burger, M. and Knyszynski, A. (1974) Inhibition of radiation-induced lymphatic leukemia in C57BL mice by 195 alpha-2-Globulin (α_2-MG) from human blood serum. Radiat. Res. 60: 501–505.

Beutler, B. and Cerami, A. (1988) The common mediator of shock, cachexia and tumor necrosis. Adv. Immunol. 42: 213–231.

Beutler, B., Milsark, I. W. and Cerami, A. (1985) Cachectin/tumor necrosis factor: production, distribution and metabolic fate in vivo. J. Immunol. 135: 3972–3977.

Billings, P. C. (1993) Approaches to studying the target enzymes of anticarcinogenic protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 191–198, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Billings, P. C. and Habres J. M. (1992) A growth regulated protease activity which is inhibited by the anticarcinogenic Bowman-Birk protease inhibitor. Proc. Natl. Acad. Sci. USA 89: 3120–3124.

Billings, P. C., Carew, J. A., Keller-McGandy, C. E., Goldberg, A. and Kennedy, A. R. (1987) A serine protease activity in C3H/10T1/2 cells that is inhibited by anticarcinogenic protease inhibitors. Proc. Natl. Acad. Sci. USA 84: 4801–4805.

Billings, P. C., St. Clair, W., Owen, A. J. and Kennedy, A. R. (1988) Potential intracellular target proteins of the anticarcinogenic Bowman-Birk protease inhibitor identified by affinity chromatography. Cancer Res. 48: 1798–1802.

Billings, P. C., Morrow, A. R., Ryan, C. A. and Kennedy, A. R. (1989) Inhibition of radiation-induced transformation of C3H/10T1/2 cells by carboxypeptidase Inhibitor I and Inhibitor II from potatoes. Carcinogenesis 10: 687–691.

Billings, P. C., Habres, J. M. and Kennedy, A. R. (1990a) Inhibition of radiation-induced transformation of C3H10T1/2 cells by specific protease substrates. Carcinogenesis 11: 329–332.

Billings, P. C., Longnecker, M. P., Keary, M. and Taylor, P. R. (1990b) Protease inhibitor content of human dietary samples. Nutr. Cancer 14: 81–93.

Billings, P. C., Newberne, P. and Kennedy, A. R. (1990c) Protease inhibitor suppression of colon and anal gland carcinogenesis induced by dimethylhydrazine. Carcinogenesis 11: 1083–1086.

Billings, P. C., Brandon, D. L. and Habres, J. M. (1991a) Internalization of the Bowman-Birk protease inhibitor by intestinal epithelial cells. Eur. J. Cancer 27: 903–908.

Billings, P. C., Habres, J. M., Liao, D. C. and Tuttle, S. W. (1991b) A protease activity in human fibroblasts which is inhibited by the anticarcinogenic Bowman-Birk protease inhibitor. Cancer Res. 51: 5539–5543.

Billings, P. C., St. Clair, W., Maki, P. A. and Kennedy, A. R. (1992) Distribution of the Bowman Birk protease inhibitor in mice following oral administration. Cancer Lett. 62: 191–197.

Billings, P. C., Maki, P. A. and Kennedy, A. R. (1994) Effect of modifiers of arachidonic acid metabolism on radiation transformation and eicosanoid formation in C3H10T1/2 cells. Cancer Biophys. Biochem. 14: 211–220.

Birk, Y. (1961) Purification and some properties of a highly active inhibitor of trypsin and chymotrypsin from soybeans. Biochim. Biophys. Acta 54: 378–381.

Birk, Y. (1974) Structure-activity relationship of several trypsin and chymotrypsin inhibitors from legume seeds. In: Proteinase Inhibitors, pp. 355–361, Fritz, H., Tschesche, H., Green, L. J. and Truscheit, E. (eds.) Springer-Verlag, Heidelberg.

Birk, Y. (1976) Proteinase inhibitors from plant sources. In: Methods in Enzymology, Vol. 45, Proteolytic Enzymes, Part B, pp. 695–739, Lorand, L. (ed.) Academic Press, New York.

Birk, Y. (1985) The Bowman-Birk Inhibitor. Int. J. Pept. Protein Res. 25: 113–131.

Birk, Y. (1993) Protease inhibitors of plant origin and role of protease inhibitors in human nutrition: overview. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 97–106, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Blondell, J. M. (1988) Urban-rural factors affecting cancer mortality in Kentucky, 1950–1969. Cancer Detect. Prev. 11: 209–223.

Bodwell, C. E. and Hopkins, D. T. (1985) Nutritional characteristics of oilseed proteins. In: New Protein Foods, Vol. 5, Seed Storage Proteins, Ch. VII, pp. 221–257, Altschul, A. A. and Wilcke H. L. (eds.) Academic Press, Orlando.

Bohe, M. (1987) Pancreatic and granulocytic endoproteases in faecal extracts of patients with active ulcerative colitis. Scand. J. Gastroenterol. 22: 59–64.

Bohe, M., Genell, S. and Ohlsson, K. (1986) Protease inhibitors in plasma and faecal extracts from patients with active inflammatory bowel disease. Scand. J. Gastroenterol. 21: 598–604.

Boone, C. W. and Kelloff, G. J. (1994) Development of surrogate endpoint biomarkers for clinical trials of cancer chemopreventive agents: relationships to fundamental properties of preinvasive (intraepithelial) neoplasia. J. Cell. Biochem. 19 (Suppl.): 10–22.

Boorman, G. A. and Eustis, S. L. (1984) Proliferative lesions of the exocrine pancreas in male F344/N rats. Environ. Health Perspect. 56: 213–214.

Boorman, G. A., Banas, D. A., Eustis, S. L. and Haseman, J. K. (1987) Proliferative exocrine pancreatic lesions in rats. The

effect of sample size on the incidence of lesions. Toxicol. Pathol. 15: 451–456.

Borek, C. and Cleaver, J. E. (1981) Protease inhibitors neither damage DNA nor interfere with DNA repair or replication in human cells. Mutat. Res. 82: 373–380.

Borek, C., Miller, R. C., Pain, C. and Troll, W. (1979) Conditions for inhibiting and enhancing effects of the protease inhibitor antipain on x-ray-induced neoplastic transformation in hamster and mouse cells. Proc. Natl. Acad. Sci. USA 76: 1800–1803. Erratum: Proc. Natl. Acad. Sci. USA 76: 6699 (1979).

Bowman, D. E. (1946) Differentiation of soybean antitryptic factors. Proc. Soc. Exp. Biol. Med. 63: 547–550.

Bowman, D. E. (1993) Discovery and background of the Bowman-Birk protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 93–96, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Bozzini, A. and Silano, V. (1978) Control through breeding methods of factors affecting nutritional quality of cereals and grain legumes. In: Nutritional Improvement of Food and Feed Proteins, pp. 249–274, Friedman, M. (ed.) Plenum Press, New York.

Brandon, D. L., Bates, A. H. and Friedman, M. (1989) Monoclonal antibody-based enzyme immunoassay of the Bowman-Birk protease inhibitor of soybeans. Agric. Food Chem. 37: 1192–1196.

Brent, R. L. (1978) Vulnerability of the preimplantation mammalian embryo. Pediatr. Res. 12: 392.

Brown, W. and Ryan, C. A. (1984) Isolation and characterization of a wound-induced trypsin inhibitor from alfalfa leaves. Biochemistry 23: 3418–3422.

Buell, P. (1973) Changing incidence of breast cancer in Japanese-American women. J. Natl. Cancer Inst. 51: 1479–1483.

Caggana, M. and Kennedy, A. R. (1989) C-fos mRNA levels are reduced in the presence of antipain and the Bowman-Birk inhibitor. Carcinogenesis 10: 2145–2148.

Calaf, G. and Russo, J. (1993) Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 14: 483–492.

Carew, J. A. and Kennedy, A. R. (1990) Identification of a proteolytic activity which responds to anticarcinogenic protease inhibitors in C3H10T1/2 cells. Cancer Lett. 49: 153–163.

Carroll, K. K. and Khor, H. T. (1975) Dietary fat in relation to tumorigenesis. Prog. Biochem. Pharmacol. 10: 307–353.

Chang, J. D. and Kennedy, A. R. (1988) Cell cycle progression of C3H10T1/2 and 3T3 cells in the absence of a transient increase in c-myc RNA levels. Carcinogenesis 9: 17–20.

Chang, J. D. and Kennedy, A. R. (1993) Suppression of c-myc by anticarcinogenic protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 265–280, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Chang, J. D., Billings, P. and Kennedy, A. R. (1985) C-myc expression is reduced in antipain-treated proliferating C3H10T1/2 cells. Biochem. Biophys. Res. Commun. 133: 830–835.

Chang, J. D., Li, J-H., Billings, P. C. and Kennedy, A. R. (1990) Effects of protease inhibitors on c-myc expression in normal and transformed C3H10T1/2 cells. Mol. Carcinog. 3: 226–232.

Chapman, H. A., Jr. and Stone, O. L. (1984) Comparison of live human neutrophil and alveolar macrophage elastolytic activity in vitro: relative resistance of macrophage elastolytic activity to serum and alveolar proteinase inhibitors. J. Clin. Invest. 74: 1693–1700.

Chernick, S. S., Lepkovsky, S. and Chaikoff, I. L. (1948) A

dietary factor regulating the enzyme content of the pancreas: changes induced in size and proteolytic activity of the chick pancreas by the ingestion of raw soybean meal. Am. J. Physiol. 155: 33–41.

Churella, H. R., Yao, B. C. and Thompson, W. A. B. (1976) Soybean trypsin inhibitor activity of soy infant formulas and its nutritional significance for the rat. J. Agric. Food Chem. 24: 393–397.

Clark, D. A., Day, R., Seidah, N., Moody, T. W., Cuttitta, F. and Davis, T. P. (1993) Protease inhibitors suppress in vitro growth of human small cell lung cancer. Peptides 14: 1021–1028.

Clifton, K. H., Domann, F. E. and Groch, K. M. (1991) On the cells of origin of radiogenic thyroid cancer: new studies based on an old idea. J. Radiat. Res. 2 (Suppl.): 143–155.

Coburn, M., Schuel, H. and Troll, W. (1981) A hydrogen peroxide block to polyspermy in the sea urchin, Arbacia punctulata. Dev. Biol. 84: 235–238.

Corasanti, J. G., Hobika, G. H. and Markus, G. (1982) Interference with dimethylhydrazine induction of colon tumors in mice by ε-aminocaproic acid. Science 216: 1020–1021.

Correa, P. (1981) Epidemiologic correlations between diet and cancer frequency. Cancer Res. 41: 3685–3690.

Corwin, L. M. and Gordon, R. K. (1982) Vitamin E and immune regulation. Ann. NY Acad. Sci. 393: 437–451.

Crass, R. A. and Morgan, R. G. H. (1982) The effect of long-term feeding of soya bean flour diets on pancreatic growth in the rat. Br. J. Nutr. 47: 119–129.

Das, K. M. (1991) Some current perspectives on the etiology and pathogenesis of ulcerative colitis. In: Ulcerative Colitis, pp. 47–54, O'Morain, C. A. (ed.) CRC Press, Boca Raton.

Dayal, Y. and DeLellis, R. A. (1989) The gastrointestinal tract. In: Robbins Pathologic Basis of Disease, 4th edn., pp. 827–910, Cotran, R. S., Kumar, V. and Robbins, S. L. (eds.) W. B. Saunders Co., Philadelphia.

Diaz, G. R., Devaux, M. A., Johnson, C. D., Adrich, Z. and Sarles, H. (1982) Physiological conditions for the study of basal and meal-stimulated exocrine pancreatic secretion in the dog. Absence of feedback inhibition of basal secretion. Can. J. Physiol. Pharmacol. 60: 1287–1295.

DiPaolo, J. A., Amsbaugh, S. C. and Popescu, N. C. (1980) Antipain inhibits N-methyl-N'-nitro-N-nitrosoguanidine-induced transformation and increases chromosomal aberrations. Proc. Natl. Acad. Sci. USA 77: 6649–6653.

Dittmann, K., Loffler, H., Bamberg, M. and Rodemann, H. P. (1995) Bowman-Birk proteinase inhibitor (BBI) modulates radiosensitivity and radiation-induced differentiation of human fibroblasts in culture. Radiother. Oncol. 34: 137–143.

Doell, B. H., Ebden, C. J. and Smith, C. A. (1981) Trypsin inhibitor activity of conventional foods which are part of the British diet and some soya products. Qual. Plant. Plant Foods Hum. Nutr. 31: 139–150.

Doll, R. and Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J. Natl. Cancer Inst. 66: 1193–1308.

Douglas, B. R., Woutersen, R. A., Jansen, J. B. M. J., de Jong, A. J. L., Rovati, L. C. and Lamers, C. B. H. W. (1989) Modulation by CR-1409 (Lorglumide), a cholecystokinin receptor antagonist, of trypsin inhibitor-enhanced growth of azaserine-induced putative preneoplastic lesions in rat pancreas. Cancer Res. 49: 2438–2441.

Dunn, J. E. (1975) Cancer epidemiology in populations of the United States with emphasis on Hawaii and California and Japan. Cancer Res. 35: 3240–3245.

Durbec, J. P., Chevilotte, G., Bidart, J. M., Berthezene, P. and Sarels, H. (1983) Diet, alcohol, tobacco and risk of cancer of the pancreas—a case control study. Br. J. Cancer 47: 463–470.

Eisen, V. and Wilson, C. (1957) The effect of β-irradiation on skin histamine and vascular responses in the rat. J. Physiol. 136: 122–139.

Ekrami, H., Kennedy, A. R., Witschi, H. and Shen, W. C. (1993) Cationized Bowman-Birk protease inhibitor as a targeted cancer chemopreventive agent. J. Drug Target. 1: 41–49.

Ekrami, H., Kennedy, A. R. and Shen, W. C. (1995a) Disposition of positively-charged Bowman-Birk protease inhibitor conjugates in mice: influence of protein conjugate charge-density and size on lung targeting. J. Pharm. Sci. 84: 456–461.

Ekrami, H. M., Kennedy, A. R. and Shen, W. C. (1995b) Water-soluble fatty acid derivatives as acylating agents for reversible lipidization of polypeptides. FEBS Lett. 371: 283–286.

Ethier, S. P. and Ullrich, R. L. (1982) Detection of ductal dysplasia in mammary outgrowths derived from carcinogen-treated virgin female BALB/c mice. Cancer Res. 42: 1753–1760.

Evans, S. M., Szuhaj, B. F., Van Winkle, T., Michel, K. and Kennedy, A. R. (1992) Protection against metastasis of radiation induced thymic lymphosarcoma and weight loss in C57B1/6NCr1BR mice by an autoclave resistant factor present in soybeans. Radiat. Res. 132: 259–262.

Fabre, F. and Roman, H. (1977) Genetic evidence for inducibility of recombination competence in yeast. Proc. Natl. Acad. Sci. USA 74: 1667–1671.

Fahmy, M. J. and Fahmy, O. G. (1980) Intervening DNA insertions and the alteration of gene expression by carcinogens. Cancer Res. 40: 3374–3382.

Flavin, D. F. (1982) The effect of soybean trypsin inhibitors on the pancreas of animals and man. Vet. Hum. Toxicol. 24: 25–28.

Flick, M. B. and Kennedy, A. R. (1991) Effect of protease inhibitors on DNA amplification in SV40-transformed Chinese hamster embryo cells. Cancer Lett. 56: 102–108.

Folsch, U. R., Winckler, K. and Wormsley, K. G. (1974) Effect of a soybean diet on enzyme content and ultrastructure of the rat exocrine pancreas. Digestion 11: 161–171.

Fontham, E. and Correa, P. (1993) The epidemiologic approach to the study of protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 1–8, Troll, W. and Kennedy, A. (eds.) Plenum Publishing Corporation, New York and London.

Ford, J. E. (1981) Microbiological methods for protein quality assessment. In: Protein Quality in Humans: Assessment and In Vitro Estimation, pp. 278–301, Bodwell, C. E., Adkins, J. S. and Hopkins, D. T. (eds.) AVI Publishing Company, Inc., Westport.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage. Pharmacol. Ther. 53: 127–166.

Frenkel, K., Chrzan, K., Ryan, C., Wiesner, R. and Troll, W. (1987) Chymotrypsin-specific protease inhibitors decrease H_2O_2 formation by activated human polymorphonuclear leukocytes. Carcinogenesis 8: 1207–1212.

Fushiki, T. and Iwai, K. (1989) Two hypotheses on the feedback regulation of pancreatic enzyme secretion. FASEB J. 3: 121–126.

Gallaher, D. and Schneeman, B. O. (1986) Nutritional and metabolic response to plant inhibitors of digestive enzymes. Adv. Exp. Med. Biol. 199: 167–184.

Garte, S. J., Currie, D. C. and Troll, W. (1987) Inhibition of H-ras oncogene transformation of NIH3T3 cells by protease inhibitors. Cancer Res. 47: 3159–3162.

German, J. (1995) Bloom's Syndrome. Dermatol. Clin. 13: 7–18.

Gertler, A. and Nitsan, Z. (1970) The effect of trypsin inhibitors on pancreotopeptidase E, trypsin, chymotrypsin, and amylase in the pancreas and intestinal tract of chicks receiving raw and heated soya bean diets. B. J. Nutr. 24: 803–804.

Gold, E. B., Gordis, L., Diener, M. D., Seltser, R., Boitnott, J. K., Bynum, T. E. and Hutcheon, D. F. (1985) Diet and other risk factors for cancer of the pancreas. Cancer 55: 460–467.

Goldfarb, R. H., Kitson, R. P., Giffen, C. Z. and Yavelow, J. (1989) Anticarcinogenic and anti-proteolytic Bowman-Birk Inhibitor (BBI): failure to inhibit both LAK cell proteases and LAK cell-mediated killing of tumor cells. Proc. Am. Assoc. Cancer Res. 30: 179.

Goldstein, B. D., Witz, G., Amoruso, M. and Troll, W. (1979) Protease inhibitors antagonize the activation of polymorphonuclear leukocyte oxygen consumption. Biochem. Biophys. Res. Commun. 88: 854–860.

Gorrill, A. D. L. and Thomas, J. W. (1967) Body weight changes, pancreas size and enzyme activity, and proteolytic enzyme activity and protein digestion in intestinal contents from calves fed soybean and milk protein diets. J. Nutr. 92: 215–223.

Gottesman, M. N. (1987) Regulation by proteolysis. In: Escherichia coli and Salmonella typhimurium, pp. 1308–1312, Neidhardt, F. (ed.) American Society for Microbiology, Washington, DC.

Grau, C. R. and Carroll, R. W. (1958) Evaluation of protein quality. In: Processed Plant Protein Foodstuffs, Ch. 7, pp. 153–189, Altschul, A. M. (ed.) Academic Press, New York.

Green, G. M. and Lyman, R. L. (1972) Feedback regulation of pancreatic enzyme secretion as a mechanism for trypsin inhibitor induced hypersecretion in rats. Proc. Soc. Exp. Biol. Med. 140: 6–12.

Green, G. M., Olds, B. O., Matthews, G. and Lyman, R. L. (1973) Protein as a regulator of pancreatic enzyme secretion in the rat. Proc. Soc. Exp. Biol. Med. 142: 1162–1167.

Green, T. R. and Ryan, C. A. (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777.

Grobstein, C., Cairns, J., Broitman, S. A., Campbell, T. C., Gussow, J. D., Kolonel, L. N., Kritchevsky, D., Mertz, W., Miller, A. B., Prival, M. J., Slaga, T., Wattenberg, L. and Sugimura, T. (1982) Diet, Nutrition and Cancer. Committee on Diet, Nutrition and Cancer, pp. 1-1–18-16, Assembly of Life Sciences, National Research Council, National Academy of Sciences, Washington, DC.

Gumbmann, M. R., Spangler, W. L., Dugan, G. M., Rackis, J. J. and Liener, I. E. (1985) The USDA trypsin inhibitor study, IV: the chronic effects of soyflour and soy protein isolate in rats after two years. Qual. Plant Food Hum. Nutr. 35: 275–314.

Gumbmann, M. R., Dugan, G. M., Spangler, W. L., Barker, E. C. and Rackis, J. J. (1989) Pancreatic response in rats and mice to trypsin inhibitors from soy and potato after short and long term dietary exposure. J. Nutr. 199: 1598–1609.

Habres, J. M. and Billings, P. C. (1992) Intestinal epithelial cells contain a high molecular weight protease subject to inhibition by anticarcinogenic protease inhibitors. Cancer Lett. 63: 135–142.

Haenszel, W. and Kurihara, M. (1968) Studies of Japanese immigrants. I. Mortality from cancer and other diseases among Japanese in the U.S. J. Natl. Cancer Inst. 40: 43–69.

Hanauer, S. B. and Baert, F. (1994) Medical therapy of inflammatory bowel disease. Med. Clin. North Am. 78: 1413–1426.

Harlan, J. M., Killen, P. D., Harker, L. A., Striker, G. E. and Wright, D. G. (1981) Neutrophil-mediated endothelial injury in vitro: mechanisms of cell detachment. J. Clin. Invest. 68: 1394–1403.

Hasdai, A. and Liener, I. E. (1983) Growth, digestibility, and enzymatic activities in the pancreas and intestines of hamsters fed raw and heated soy flour. J. Nutr. 113: 662–668.

Haseman, J. K., Huff, J. E., Rao, G. N., Arnold, J. E., Boorman, G. A. and McConnell, E. E. (1985) Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F$_1$ (B6C3F$_1$) mice. J. Natl. Cancer Inst. 75: 975–984.

Hawkins, J. V., Emmel, E. L., Feuer, J. J., Nedelman, M. A., Harvey, C. J., Klein, H. S., Rozmiarek, H., Kennedy, A. R., Lichtenstein, G. R. and Billings, P. C. (1997) Protease activity in a hapten-induced model of ulcerative colitis in rats. Dig. Dis. 42: 1969–1980.

Heilbronn, R., Schlehofer, J. R., Yalkinoglu, A. O. and Zur Hausen, H. (1985) Selective DNA amplification induced by carcinogens (initiators): evidence for a role of proteases and DNA polymerase alpha. Int. J. Cancer 36: 85–91.

Hirahara, F., Miyagi, Y., Miyagi, E., Yasumitsu, H., Koshikawa, N., Nagashima, Y., Kitamura, H., Minaguchi, H., Umeda, M. and Miyazaki, K. (1995) Trypsinogen expression in human ovarian carcinomas. Int. J. Cancer 63: 176–181.

Hooks, R. D., Hays, V. W., Speer, V. C. and McCall, J. T. (1965) Effects of raw soybeans on pancreatic enzyme concentrations and performance of pigs. Fed. Proc. Fed. Am. Soc. Exp. Biol. 24: 894.

Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, G. M., Mirand, E. A. and Murphy, G. P. (1983) LNCaP model of human prostatic carcinoma. Cancer Res. 43: 1809–1818.

Hozumi, M., Ogawa, M., Sugimura, T., Takeuchi, T. and Umezawa, H. (1972) Inhibition of tumorigenesis in mouse skin by leupeptin, a protease inhibitor from actinomycetes. Cancer Res. 32: 1725–1728.

Hwang, D. L. R., Davis-Lin, K. T., Yang, W. K. and Foard, D. T. (1977) Purification, partial characterization and immunological relationships of multiple low molecular weight proteinase inhibitors of soybean. Biochem. Biophys. Acta 495: 369–382.

Hu, E., Mueller, E., Oliviero, S., Papaioannou, V. E., Johnson, A. and Spiegelman, B. M. (1994) Targeted disruption of the c-fos gene demonstrates c-fos-dependent and -independent pathways for gene expression stimulated by growth factors or oncogenes. EMBO J. 13: 3094–3103.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) Studies on a cyclid nucleotide-independent protein kinase and its proenzyme in mammalian tissues. J. Biol. Chem. 252: 7610–7616.

Ishiura, S. (1981) Calcium-dependent neutral protease from chicken skeletal muscle. I. Purification and characterization. J. Biochem. 84: 225–230.

Jochum, M., Witte, J., Schiessler, H. and Fritz, H. (1981) Clotting and other plasma factors in septicemia: inhibition of degradation by an exogenous protease inhibitor from soybeans (type: Bowman-Birk). B.7. Fibrinolytic system. Protids Biol. Fluids 28: 417–420.

Jochum, M., Duswald, K.-H., Neumann, S., Witte, J. and Fritz, H. (1984) Poteinases and their inhibitors in septicemia—basic concepts and clinical implications. Adv. Exp. Med. Biol. 167: 391–404.

Kakade, M. L., Barton, T. L., Schaible, P. J. and Evan, R. J. (1967)

Biochemical changes in the pancreas of chicks fed raw soybeans and soybean meal. Poult. Sci. 46: 1578–1585.

Kakade, M. L., Hoffa, D. E. and Liener, I. E. (1973) Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. J. Nutr. 103: 1772–1778.

Kakade, M. L., Thompson, R. D., Englestad, W. W., Behrens, G. C., Yoder, R. D. and Crave, F. M. (1976) Failure of soybean trypsin inhibitors to exert deleterious effects in calves. J. Dairy Sci. 59: 1484–1489.

Kamiya, K., Yasukawa-Barnes, J., Mitchen, J. M., Gould, M. N. and Clifton, K. H. (1995) Evidence that carcinogenesis involves an imbalance between epigenetic high-frequency initiation and suppression of promotion. Proc. Natl. Acad. Sci. USA 92: 1332–1336.

Kassell, B. (1970) Trypsin and chymotrypsin inhibitors from soybeans. Methods Enzymol. 19: 853–862.

Kelenyi, G. (1953) Changes in mast cells following x-irradiation. Acta Morphol. 3: 377–381.

Kelloff, G. J., Johnson, J. R., Crowell,. J. A., Boone, C. W., DeGeorge, J. J., Steele, V. E., Mehta, M. U., Temeck, J. W., Schmidt, W. J., Burke, G., Greenwald, P. and Temple, R. J. (1995) Approaches to the development and marketing approval of drugs that prevent cancer. Cancer Epidemiol. Biomarkers Prev. 4: 1–10.

Kennedy, A. R. (1982) Antipain, but not cycloheximide, suppresses radiation transformation when present for only one day at five days post-irradiation. Carcinogenesis 3: 1093–1095.

Kennedy, A. R. (1984) Promotion and other interactions between agents in the induction of transformation in vitro in fibroblasts. In: Mechanisms of Tumor Promotion, Vol. III, Tumor Promotion and Carcinogenesis In Vitro, Ch. 2, pp. 13–55, Slaga, T. J. (ed.) CRC Press, Inc., Boca Raton.

Kennedy, A. R. (1985a) The conditions for the modification of radiation transformation in vitro by a tumor promoter and protease inhibitors. Carcinogenesis 6: 1441–1446.

Kennedy, A. R. (1985b) Evidence that the first step leading to carcinogen-induced malignant transformation is a high frequency, common event. In: Carcinogenesis; A Comprehensive Survey, Vol. 9, Mammalian Cell Transformation: Mechanisms of Carcinogenesis and Assays for Carcinogens, pp. 355–364, Barrett, J. C. and Tennant, R. W. (eds.) Raven Press, New York.

Kennedy, A. R. (1988) Implications for mechanisms of tumor promotion and its inhibition by various agents from studies of in vitro transformation. In: Tumor Promoters, Biological Approaches for Mechanistic Studies and Assay Systems, pp. 201–212, Langenbach, R., Barrett, J. C. and Elmore, E. (eds.) Raven Press, New York.

Kennedy, A. R. (1989) Initiation and promotion of radiation induced transformation in vitro: relevance of in vitro studies to radiation induced cancer in human populations. In: Cell Transformation and Radiation-Induced Cancer, Ch. 7, pp. 263–270, Chadwick, K. H., Seymour, C. and Barnhart, B. (eds.) IOP Publishing, Ltd (Adam Hilger), Bristol.

Kennedy, A. R. (1990) Effects of protease inhibitors and vitamin E in the prevention of cancer. In: Nutrients and Cancer Prevention, pp. 79–98, Prasad, K. N. and Meyskens, F. L., Jr. (eds.) The Humana Press, Inc., Clifton.

Kennedy, A. R. (1991) Is there a critical target gene for the first step in carcinogenesis? Environ. Health Perspect. 93: 199–203.

Kennedy, A. R. (1993a) Overview: anticarcinogenic activity of protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 9–64, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Kennedy, A. R. (1993b) In vitro studies of anticarcinogenic protease inhibitors. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 65–91, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Kennedy, A. R. (1993c) Cancer prevention by protease inhibitors. Prev. Med. 22: 796–811.

Kennedy, A. R. (1993d) The impact of protease inhibitors from soybeans on the carcinogenic process. In: Adjuvant Nutrition in Cancer Treatment, pp. 129–144, Quillin, P. and Williams. R. M. (eds.) Cancer Treatment Research Foundation, Arlington Heights.

Kennedy, A. R. (1993e) Potential mechanisms of antitumorigenesis by protease inhibitors. In: Antimutagenesis and Anticarcinogenesis: Mechanisms, Vol. 3, pp. 301–308, Bronzetti, G., Hayatsu, A., DeFlora, S., Waters, M. D. and Shankel, D. M. (eds.) Plenum Publishing Corporation, New York and London.

Kennedy, A. R. (1993f) Use of cell and organ cultures in the identification and characterization of agents that modify carcinogenesis. Cancer Res. 53 (Suppl.): 2446s–2448s.

Kennedy, A. R. (1994) Prevention of carcinogenesis by protease inhibitors. Cancer Res. 54 (Suppl.): 1999s–2005s.

Kennedy, A. R. (1995a) The evidence for soybean products as cancer preventive agents. J. Nutr. 125 (Suppl.): 733s–743s.

Kennedy, A. R. (1995b) Mechanisms of cancer prevention: effects of protease inhibitors on proteases and gene expression. In: Nutrients in Cancer Prevention and Treatment, pp. 71–82, Prasad, K. N., Santamaria, L. and Williams, R. M. (eds.) The Humana Press, Inc., Clifton.

Kennedy, A. R. (1996a) Letter to the editor. J. Nutr. 126: 584–585.

Kennedy, A. R. (1996b) Is a mutagenic event involved in radiation induced malignant transformation? Mutat. Res. 350: 81–91.

Kennedy, A. R. and Billings, P. C. (1987) Anticarcinogenic actions of protease inhibitors. In: Anticarcinogenesis and Radiation Protection, pp. 285–295, Cerutti, P. A., Nygaard, O. F. and Siomic, M. G. (eds.) Plenum Press, New York.

Kennedy, A. R. and Little, J. B. (1978) Protease inhibitors suppress radiation induced malignant transformation in vitro. Nature 276: 825–826.

Kennedy, A. R. and Little, J. B. (1980) Investigation of the mechanism for the enhancement of radiation transformation in vitro by TPA. Carcinogenesis 1: 1039–1047.

Kennedy, A. R. and Little, J. B. (1981a) Effects of protease inhibitors on radiation transformation in vitro. Cancer Res. 41: 2103–2108.

Kennedy, A. R. and Little, J. B. (1981b) High efficiency, kinetics and numerology of transformation by radiation in vitro. In: Achievements, Challenges and Prospects for the 1980's, Vol. 1, pp. 491–500, Burchenal, J. H. and Oettgen, J. F. (eds.) Grune and Stratton Inc., New York.

Kennedy, A. R. and Little, J. B. (1984) Evidence that a second event in X-ray induced oncogenic transformation in vitro occurs during cellular proliferation. Radiat. Res. 99: 228–248.

Kennedy, A. R. and Manzone, H. (1995) Effects of protease inhibitors on levels of proteolytic activity in normal and pre-malignant cells and tissues. J. Cell. Biochem. 22 (Suppl.): 188–194.

Kennedy, A. R. and Weichselbaum, R. R. (1981) Effects of 17-β-estradiol on radiation transformation in vitro: inhibition of effects by protease inhibitors. Carcinogenesis 2: 67–79.

Kennedy, A. R., Fox, M., Murphy, G. and Little, J. B. (1980) Relationship between X-ray exposure and malignant transformation in C3H 10T1/2 cells. Proc. Natl. Acad. Sci. USA 77: 7262–7266.

Kennedy, A. R., Cairns, J. and Little, J. B. (1984a) Timing of the steps in transformation of C3H10T1/2 cells by X-irradiation. Nature 307: 85–86.

Kennedy, A. R., Radner, B. and Nagasawa, H. (1984b) Protease inhibitors reduce the frequency of spontaneous chromosome abnormalities in cells from patients with Bloom syndrome. Proc. Natl. Acad. Sci. USA 81: 1827–1830.

Kennedy, A. R., Billings, P. C., Maki, P. A. and Newberne, P. (1993a) Effects of various protease inhibitor preparations on oral carcinogenesis in hamsters induced by 7,12-dimethyl-benz(a)anthracene. Nutr. Cancer 19: 191–200.

Kennedy, A. R., Szuhaj, B. F., Newberne, P. M. and Billings, P. C. (1993b) Preparation and production of a cancer chemopreventive agent, Bowman-Birk inhibitor concentrate. Nutr. Cancer 19: 281–302.

Kennedy, A. R., Beazer-Barclay, Y., Kinzler, K. W. and Newberne, P. M. (1996) Suppression of carcinogenesis in the intestines of Min mice by the soybean-derived Bowman-Birk inhibitor. Cancer Res. 56: 679–682.

Kennedy, C. W., Donahue, J. J. and Wan, X. S. (1996) Effects of the Bowman-Birk protease inhibitor on survival of fibroblasts and cancer cells exposed to radiation and cis-platinum. Nutr. Cancer 26: 209–217.

Kilpatrick, L., Johnson, J. L., Nickbarg, E. B., Wang, Z., Clifford, T. F., Banach, M., Cooperman, B. S. and Rubin, H. (1991) Inhibition of human neutrophil superoxide generation by alpha₁-antichymotryspin. J. Immunol. 146: 2388–2393.

Kim, J., Baker, D. G., Hahn, S. S., Goodchild, N. T. and Constable, W. C. (1983) Topical use of N-acetylcysteine for reduction of skin reaction to radiation therapy. Semin. Oncol. 10: 86–92.

Korbelik, M., Osmak, M., Suhar, A., Skrk, J., Turk, V. and Petrovic, D. (1988) Modification of potentially lethal damage repair by some intrinsic intra- and extracellular agents: I. Proteinases and proteinase inhibitors. Int. J. Radiat. Biol. 54: 461–474.

Koshikawa, N., Yasumitsu, H., Nagashima, Y., Umeda, M. and Miyazaki, K. (1994) Identification of one- and two-chain forms of trypsinogen 1 produced by a human gastric adenocarcinoma cell line. Biochem. J. 303: 187–190.

Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. J. Gen. Physiol. 30: 291–310.

Kuroki, T. and Drevon, C. (1979) Inhibition of chemical transformation in C3H/10T1/2 cells by protease inhibitors. Cancer Res. 39: 2755–2761.

Laine, A., Davril, M. and Hayem, A. (1982) Interaction between human serum α₁-antichymotrypsin and human leukocyte cathepsin G: complex formation and production of a modified inhibitor. Biochem. Biophys. Res. Commun. 105: 186–193.

Land, H., Parada, L. F. and Weinberg, R. A. (1983a) Cellular oncogenes and multistep carcinogenesis. Science 222: 771–778.

Land, H., Prada, L. F. and Weinberg, R. A. (1983b) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304: 596–602.

Larionova, N. I., Gladysheva, I. P., Tikhonova, T. V. and Kazanskaya, N. F. (1994a) Inhibition of cathepsin G and human granulocyte elastase by multiple forms of Bowman-Birk type soybean inhibitor. (translated from Russian) Biokhimiya 58: 1437–1444 (1993).

Larionova, N. I., Topchieva, I. N., Gladysheva, I. P. and Kennedy A. R. (1994b) The Bowman-Birk inhibitor-polyester conjugate: synthesis, inhibition of serine proteinases and suppression of radiation transformation in vitro. Cancer J. 7: 158–163.

Lavi, S. (1981) Carcinogen-mediated amplification of viral DNA

sequences in Simian virus 40-transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA 78: 6144–6148.

Lavi, S. (1986) Carcinogen-mediated amplification of specific DNA sequence. J. Cell. Biochem. 18: 149–156.

Leb, L., Beatson, P., Fortier, N., Newburger, P. E. and Snyder, L. M. (1985) Modulation of mononuclear phagocyte cytotoxicity by alpha-tocopherol (vitamin E). J. Leukoc. Biol. 37: 449–459.

Lee, H. P., Gourley, L., Duffy, S. W., Esteve, J., Lee, J. and Day, N. E. (1991) Dietary effects of breast-cancer risk in Singapore. Lancet 337: 1197–1200.

Lee, J. K., Park, B. J., Yoo, K. Y. and Ahn, Y. O. (1995) Dietary factors and stomach cancer: a case-control study in Korea. Int J. Epidemiol. 24: 33–41.

Lee, J. S., Brown, W. E., Pearce, G., Dreher, T. W., Graham, J. S., Ahern, K. G., Pearson, G. D. and Ryan, C. A. (1986) Molecular characterization and phylogenetic studies with a wound-inducible protease inhibitor gene in Lycopersicum species. Proc. Natl. Acad. Sci. USA 83: 7277–7281.

Li, J. H., Billings, P. C. and Kennedy, A. R. (1992) Induction of oncogene expression by sodium arsenite in C3H/10T1/2 cells; inhibition of c-myc expression by protease inhibitors. Cancer J. 5: 354–358.

Liddle, R. A., Goldfine, I. D. and Williams, J. A. (1984) Bioassay of plasma cholecystokinin in rats: effects of food, trypsin inhibitor and alcohol. Gastroenterology 87: 542–549.

Liebner, E. J., Moos, W. S., Hochhauser, M. and Harvey, R. A (1962) Lowering the skin temperature of the irradiated field. Am. J. Roentgenol. Radium Ther. Nucl. Med. 88: 976–987.

Liener, I. E. (1979a) Significance for humans of biologically active factors in soybeans and other food legumes. J. Am. Oil Chemists Soc. 56: 121–129.

Liener, I. E. (1979b) Protease inhibitors and lectins. In: International Review of Biochemistry, pp. 97–122, Veuberger, A. and Jakes, T. H. (eds.) University Park Press, Baltimore.

Liener, I. E. (1989) Control of anti-nutritional and toxic factors in oilseeds and legumes. In: Food Uses of Whole Oil and Protein Seeds, Ch. 22, pp. 344–371, Lusas, E. W., Erikson, D. R. and Nip, W.-K. (eds.) American Oil Chemists Society, Champaign.

Liener, I. E. and Hasdai, A. (1986) The effect of long-term feeding of raw soy flour on the pancreas of mouse and hamster. Adv. Exp. Med. Biol. 199: 189–197.

Liener, I. E. and Kakade, M. L. (1980) Protease inhibitors. In: Toxic Constituents of Plant Foodstuffs, pp. 7–71. Liener, I. E. (ed.) Academic Press, New York.

Liener, I. E., Goodale, R. L., Deshmukh, A., Satterberg, T. L., Ward, G., Dipietro, C. M., Bankey, P. E. and Borner, J. W. (1988) Effect of a trypsin inhibitor from soybeans (Bowman-Birk) on the secretory activity of the human pancreas. Gastroenterology 94: 419–427.

Lim, T. S., Putt, N., Safranski, D., Chung, C. and Watson, R. R. (1981) Effect of vitamin E on cell-mediated immune responses and serum corticosterone in young and maturing mice. Immunology 44: 289–295.

Lippman, S. M., Lee, J. S., Lotan, R., Hittelman, W., Wargovich, M. J. and Hong, W. K. (1990) Biomarkers as intermediate endpoints in chemoprevention trials. J. Natl. Cancer Inst. 82: 555–560.

Little, J. W., Edmiston, S. H., Pacelli, L. Z. and Mount, D. W. (1980) Cleavage of the Escherichia coli lexA protein by the recA protease. Proc. Natl. Acad. Sci. USA 77: 3225–3229.

Long, S., Quigley, J., Troll, W. and Kennedy, A. R. (1981) Protease inhibitor antipain suppresses TPA induction of plasmino-

gen activator in transformable mouse embryo fibroblasts. Carcinogenesis 2: 933–936.

Lu, L.-J. W., Anderson, K. E., Gomez, G. and Nealon, W. H. (1995) Decreased plasma levels of cholecystokinin in healthy males after chronic ingestion of a heat-treated soya product. Cancer Lett. 90: 149–155.

Lucibello, F. C. and Muller, R. (1991) Proto-oncogenes encoding transcriptional regulations: unravelling the mechanisms of oncogenic conversion. Crit. Rev. Oncog. 2: 259–276.

Mack, T. M. (1982) Pancreas. In: Cancer Epidemiology and Prevention, pp. 638–667, Schottenfeld, D. and Fraumeni, J. (eds.) W. B. Saunders Co., Philadelphia.

Mackay, A. A., Ballin, M., Pelina, M. D., Farina, A. R., Nason, A. M., Hartzler, J. L. and Thorgeirsson, U. P. (1992) Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of metalloproteinase expression in tumor and normal cell lines. Invasion Metastasis 12: 168–184.

Madar, Z., Birk, Y. and Gertler, A. (1974) Native and modified Bowman-Birk trypsin inhibitor—comparative effect on pancreatic enzymes upon ingestion by quails. Comp. Biochem. Physiol. 48B: 251–256.

Madar, Z., Gertler, A. and Birk, Y. (1979) The fate of the Bowman-Birk trypsin inhibitor from soybeans in the digestive tract of chicks. Comp. Biochem. Physiol. 62: 1057–1061.

Mainz, D. L., Black, O. and Webster, P. D. (1973) Hormonal control of pancreatic growth. J. Clin. Invest. 52: 2300–2304.

Maki, P. A. and Kennedy, A. R. (1992) Humoral and cellular immune functions are not compromised by the anticarcinogenic Bowman-Birk inhibitor. Nutr. Cancer 18: 165–173.

Maki, P., Paterson, Y. and Kennedy, A. R. (1994) Studies related to the potential antigenicity of the Bowman-Birk inhibitor, an anticarcinogenic protease inhibitor isolated from soybeans. Nutr. Cancer 22: 185–193.

Manzone, H., Billings, P. C., Cummings, W. N., Feldman, R., Clark, L. C., Odell, C. S., Horan, A., Atiba, J. O., Meyskens, F. L., Jr. and Kennedy, A. R. (1995) Levels of proteolytic activities as intermediate marker endpoints in oral carcinogenesis. Cancer Epidemiol. Biomarkers Prev. 4: 521–527.

Matrisian, L. M., Glaichenhaus, N., Gesnel, M. C. and Breathnach, R. (1986) Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. 6: 1679–1686.

Matsushima, T., Kakizoe, T., Kawachi, T., Hara, K., Suzimura, T., Takeuchi, T. and Umezawa, H. (1976) Effects of protease inhibitors of microbiol origin on experimental carcinogenesis. In: Fundamentals in Cancer Prevention, pp. 57–69, Magee, P. N., Takayama, S., Sugimura, T. and Matsushima, T. (eds.) University Park Press, Baltimore.

McGuiness, E. E., Morgan, R. G. H., Levison, D. A., Frape, D. L., Hopwood, D. and Wormsley, K. G. (1980) The effect of long-term feeding of soya flour on the rat pancreas. J. Scand. Gastroenterol. 15: 497–502.

McGuiness, E. E., Morgan, R. G. H. and Wormsley, K. G. (1984) Effects of soybean flour on the pancreas of rats. Environ. Health Perspect. 56: 205–212.

Melmed, R. N., El-Aaser, A. A. A. and Holt, S. J. (1976) Hypertrophy and hyperplasia of the neonatal rat exocrine pancreas induced by orally administered soybean trypsin inhibitor. Biochem. Biophys. Acta 421: 280–282.

Messadi, D. V., Billings, P., Shklar, G. and Kennedy, A. R. (1986) Inhibition of oral carcinogenesis by a protease inhibitor. J. Natl. Cancer Inst. 76: 447–452.

Messina, M. J. (ed.) (1995) Proceedings of the First International Symposium on the Role of Soy in Preventing and Treating Chronic Disease, Mesa, AZ, Feb. 20–24, 1991. J. Nutr. 125 (Suppl.).

Messina, M. and Barnes, S. (1991) The role of soy products in reducing risk of cancer. J. Natl. Cancer Inst. 83: 541–546.

Messina, M. J., Persky, V., Setchell, K. D. R. and Barnes, S. (1994) Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr. Cancer 21: 113–131.

Metropolitan Insurance Companies (1992) International longevity comparisons. Statistical Bulletin—Metropolitan Insurance Companies 73: 10–15.

Meyn, M. S., Rossman, T. and Troll, W. (1977) A protease inhibitor blocks SOS functions in Escherichia coli: antipain prevents λ repressor inactivation, ultraviolet mutagenesis and filamentous growth. Proc. Natl. Acad. Sci. USA 74: 1152–1156.

Mills, P. K., Beeson, W. L., Abbey, D. E., Fraser, G. E. and Phillips, R. L. (1988) Dietary habits and past medical history as related to fatal pancreas cancer risk among Adventists. Cancer (Phila.) 61: 2578–2585.

Miyasaka, K. and Green, G. M. (1984) Effect of partial exclusion of pancreatic juice on rat basal pancreatic secretion. Gastroenterology 86: 114–119.

Miyasaka, K., Nakamura, R., Funakoshi, A. and Kitani, K. (1989) Stimulatory effect of monitor peptide and human pancreatic secretory trypsin inhibitor on pancreatic secretion and cholecystokinin release in conscious rats. Pancreas 4: 139–144.

Mizutani, H., Schechter, N. M., Lazarus, G., Black, R. A. and Kupper, T. S. (1991) Rapid and specific conversion of precursor interleukin 1β (IL-1β) to an active IL-1 species by human mast cell chymase. J. Exp. Med. 174: 821–825.

Morgan, R. G. H. (1987) Raw soy flour and pancreatic cancer in experimental animals. In: Experimental Pancreatic Carcinogenesis, pp. 159–174, Scarpelli, D. G., Reddy, J. K. and Longnecker, D. S. (eds.) CRC Press, Inc., Boca Raton.

Morgan, R. G. H., Levinson, D. A., Hopwood, D., Saunders, J. H. B. and Wormsley, K. G. (1977) Potentiation of the action of azaserine on the rat pancreas by raw soyabean flour. Cancer Lett. 3: 87–90.

Muller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature 312: 716–720.

Myers, B. A., Hathcock, J., Shiekh, N. and Roebuck, B. D. (1991) Effects of dietary soya bean trypsin inhibitor concentrate on initiation and growth of putative preneoplastic lesions in the pancreas of the rat. Food Chem. Toxicol. 29: 437–443.

Naim, M., Gertler, A. and Birk, Y. (1982) The effect of dietary raw and autoclaved soya-bean protein fractions on growth, pancreatic enlargement and pancreatic enzymes in rats. Br. J. Nutr. 47: 281–288.

National Toxicology Program (1994) Technical Report on the Comparative Toxicology Studies of Corn Oil, Safflower Oil and Tricaprylin (CAS Nos. 8001-30-7, 8001-23-8 and 538-23-8) in Male F344/N Rats as Vehicles for Gavage, National Toxicology Program, P.O. Box 12233, Research Triangle Park, NC 27709 (NTP TR 426; NIH Publication No. 94-3157).

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308: 693–698.

Nitsan, Z. and Alumot, E. (1964) Overcoming the inhibition of intestinal proteolytic activity caused by raw soybean in chicks of different ages. J. Nutr. 84: 179–184.

Nitsan, Z. and Nir, I. (1977) A comparative study of the nutritional and physiological significance of raw and heated soybeans in chicks and goslings. J. Nutr. 37: 81–91.

Nomura, T., Hata, S., Enomoto, T., Tanaka, H. and Shibata, K. (1980) Inhibiting effects of antipain on urethane induced lung neoplasia in mice. Br. J. Cancer 42: 624–626.

Noonan, N. E. and Noonan, K. D. (1977) The effect of TLCK on transcription and its role in modifying cell growth. J. Cell. Physiol. 92: 137–144.

Norell, S. E., Ahlbom, A., Erwald, R., Jacobson, G., Lindberg-Navier, I., Olin, R., Tornberg, B. and Wiechel, K. L. (1986) Diet and pancreatic cancer: a case-control study. Am. J. Epidemiol. 124: 894–902.

Odani, S. and Ikenaka, T. J. (1973) Scission of soybean Bowman-Birk proteinase inhibitor into two small fragments having either trypsin or chymotrypsin inhibitor activity. J. Biochem. (Tokyo) 74: 857–860.

Ohkoshi, M. and Fujii, S. (1983) Effect of the synthetic protease inhibitor N1N-dimethylcarbamoylmethyl 34-(4-guanidinobenzoyloxy) phenylacetate methanesulfate on carcinogenesis by 3-methylcholanthrene in mouse skin. J. Natl. Cancer Inst. 71: 1053–1057.

Ohlsen, L., Evers, H., Segerstrom, K., Hagelquist, E. and Graffman, S. (1987) Local anaesthetics modifying the dermal response of radiation. Acta Oncol. 26: 467–476.

Oreffo, V. I. C., Billings, P. C., Kennedy, A. R. and Witschi, H. (1991) Acute effects of the Bowman-Birk protease inhibitor in mice. Toxicology 69: 165–176.

Osborne, T. B. and Mendel, L. B. (1917) The use of soybean as food. J. Biol. Chem. 32: 369–387.

Palladino, M. A., Galton, J. E., Troll, W. and Thorbecke, G. J. (1982) Gamma irradiation-induced mortality: protective effect of protease inhibitors in chickens and mice. Int. J. Radiat. Biol. 41: 183–191.

Patten, J. R., Richards, E. A. and Wheeler, J. (1971a) The effect of raw soybean on the pancreas of adult dogs. Proc. Soc. Exp. Biol. Med. 137: 58.

Patten, J. R., Richards, E. A. and Wheeler, J. (1971b) The effect of dietary soybean trypsin inhibitor on the histology of dog pancreas. Life Sci. 10: 145–150.

Patten, J. R., Patten, J. A. and Pope, H. (1973) Sensitivity of the guinea pig to raw soya bean in the diet. Food Cosmet. Toxicol. 11: 577–583.

Pena, A. S., Shivanada, S. and Mayberry, J. F. (1991) Epidemiology of ulcerative colitis. In: Ulcerative Colitis, pp. 1–8, O'Morain, C. A. (ed.) CRC Press, Boca Raton.

Permert, J., Kazakoff, K., Pour, P. M. and Adrian, E. E. (1993) Effect of CCK receptor blockade on pancreatic tumor initiation in hamsters fed raw soya diet. Gastroenterology 104: A1042.

Persiani, S., Yeung, A., Shen, W. C. and Kennedy, A. R. (1991) Polylysine conjugates of Bowman-Birk protease inhibitor as targeted anticarcinogenic agents. Carcinogenesis 12: 1149–1152.

Phillips, R. L. (1975) Role of lifestyle and dietary habits in risk of cancer among Seventh Day Adventists. Cancer Res. 35: 3515–3522.

Phillips, R. L., Garfinkel, L., Kuzma, J. W., Beeson, W. L., Lotz, T. and Brin, B. (1980) Mortality among California Seventh-Day Adventists for selected cancer sites. J. Natl. Cancer Inst. 65: 1097–1107.

Pineda, O., Torun, B., Viteri, F. E. and Arroyave, G. (1982) Protein quality in relation to estimates of essential amino acid requirements. In: Protein Quality in Humans: Assessment and In Vitro Estimation, pp. 29–42, Bodwell, C. E., Adkins, J. S. and Hopkins, D. T. (eds.) AVI Publishing Co., Westport.

Potera, M. E., Lookingbill, D. P. and Stryker, J. A. (1982) Prophylaxis of radiation dermatitis with a topical cortisone cream. Radiology 143: 775–777.

Pour, P. M. (1991) Modification of tumor development in the pancreas. Prog. Exp. Tumor Res. 33: 108–131.

Rackis, J. J. and Anderson, R. L. (1964) Isolation of four soybean trypsin inhibitors by DEAE-cellulose chromatography. Biochem. Biophys. Res. Commun. 15: 230–235.

Rackis, J. J., McGhee, J. E. and Booth, A. N. (1975) Biological threshold levels of soybean trypsin inhibitor by rat bioassay. Cereal Chem. 52: 84–93.

Rackis, J. J., Wolf, W. J. and Baker, E. C. (1986) Protease inhibitors in plant foods: content and inactivation. Adv. Exp. Med. Biol. 199: 299–347.

Reznikoff, C. A., Brankow, D. W. and Heidelberger, C. (1973) Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to post-confluence inhibition of cell division. Cancer Res. 33: 3231–3238.

Richter, B. D. and Schneeman, B. O. (1987) Pancreatic response to long-term feeding of soy protein isolate, casein or egg white in rats. J. Nutr. 117: 247–252.

Robbins, D. J., Liener, I. E., Spangler, W. M. and de Bruyn, D. B. (1988) The effects of a raw soy protein diet on the pancreas of the Chacma baboon. Nutr. Rep. Int. 38: 9–16.

Roebuck, B. D. and Longnecker, D. S. (1993) Protease inhibitors and pancreatic carcinogenesis. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 295–301, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Roebuck, B. D., Kaplita, P. V., Edwards, B. R. and Praissman, M. (1987) Effects of dietary fats and soybean protein on azaserine-induced pancreatic carcinogenesis and plasma cholecystokinin in the rat. Cancer Res. 47: 1333–1338.

Rosen, A. and Klein, G. (1983) UV light-induced immunoglobulin heavy-chain class switch in a human lymphoblastoid cell line. Nature 306: 189–190.

Rossman, T. G. and Klein, C. B. (1985) Mammalian SOS system: a case of misplaced analogies. Cancer Invest. 3: 175–187.

Rossman, T. G. and Troll, W. (1980) Protease inhibitors in carcinogenesis: possible sites of action. In: Carcinogenesis: Modifiers of Chemical Carcinogenesis, Vol. 5, pp. 127–143, Slaga, T. J. (ed.) Raven Press, New York.

Rothman, S. S. and Wells, H. (1967) Enhancement of pancreatic enzyme synthesis by pancreozymin. Am. J. Physiol. 213: 215–218.

Rudolph, U., Finegold, M. J., Rich, S. S., Hairiman, G. R., Srinivasan, Y., Brabet, P., Boulay, G., Bradley, A. and Birnbaumer, L. (1995) Ulcerative colitis and adenocarcinoma of the colon in Ga_α-deficient mice. Nat. Genet. 10: 143–150.

Russo, J., Calaf, G. and Russo, I. H. (1993) A critical approach to the malignant transformation of human breast epithelial cells with chemical carcinogens. Crit. Rev. Oncog. 4: 403–417.

Ryan, C. A. and Kassell, B. (1970) Chymotrypsin inhibitor I from potatoes. Methods Enzymol. 19: 883–889.

Saarinen, J., Kalkkinen, N., Welgus, H. G. and Kovanen, P. T. (1994) Activation of human interstitial procollagenase through direct cleavage of the Leu[83]-Thr[84] bond by mast cell chymase. J. Biol. Chem. 269: 18134–18140.

Sale, J. K., Goldberg, D. M., Fawcett, A. N. and Wormsley, K. G. (1977) Chronic and acute studies indicating absence of exocrine pancreatic feedback inhibition in dogs. Digestion 15: 540–555.

Sawey, M. J., Hood, A. T., Burns, F. J. and Garte, S. J. (1987) Activation of myc and ras oncogenes in primary rat tumors induced by ionizing radiation. Mol. Cell. Biol. 7: 932–935.

Schechter, N. M., Sprows, J. L., Schoenberger, O. L., Lazarus, G. S., Cooperman, B. S. and Rubin, H. (1989) Reaction of human skin chymotrypsin-like proteinase chymase with plasma proteinase inhibitors. J. Biol. Chem. 264: 21308–21315.

Schelp, F. P. and Pongpaew, P. (1988) Protection against cancer through nutritionally-induced increase in endogenous proteinase inhibitors—a hypothesis. Int. J. Epidemiol. 17: 287–292.

Schingoethe, D. J., Aust, S. D. and Thomas, J. W. (1970) Separation of a mouse growth inhibitor in soybeans from trypsin inhibitors. J. Nutr. 100: 739–748.

Schonthal, A., Herrlich, P., Rahmsdorf, H. J. and Ponta, H. (1988) Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 54: 325–334.

Scott, R. E. and Maercklein, P. B. (1985) An initiator of carcinogenesis selectively and stably inhibits stem cell differentiation: a concept that initiation of carcinogenesis involves multiple phases. Proc. Natl. Acad. Sci. USA 82: 2995–2999.

Scrimshaw, N. S., Wayler, A. H., Murray, E., Steinke, F. H., Rand, W. M. and Young, V. R. (1983) Nitrogen balance response in young men given one or two isolated soy proteins or milk proteins. J. Nutr. 113: 2492–2497.

Senda, S., Fujiyama, Y., Bamba, T. and Hosoda, S. (1993) Treatment of ulcerative colitis with camostat mesilate, a serine protease inhibitor. Intern. Med. 32: 350–354.

Severson, R. K., Nomura, A. M. Y., Grove, J. S. and Stemmermann, G. N. (1989) A prospective study of demographics, diet and prostate cancer among men of Japanese ancestry in Hawaii. Cancer Res. 49: 1857–1860.

Shasby, D. M. (1985) Antioxidant activity of some antiproteases. Am. Rev. Resp. Dis. 131: 293–294.

Shen, W. C., Wan, J. and Shen, D. (1990) Proteolytic processing in a non-lysosomal compartment is required for transcytosis of protein-polylysine conjugates in cultured Madin-Darby canine kidney cells. Biochem. Biophys. Res. Commun. 166: 316–323.

Silverman, J. and Adams, J. O. (1983) N-Nitrosamines in laboratory animal feed and bedding. Lab. Anim. Sci. 33: 161–164.

Sipos, E. (1990) Dietary trends for vegetable proteins in foods. Inform 1: 753–757.

Slaga, T. J., Klein-Szanto, A. J. P., Fischer, S. M., Weeks, C. E., Nelson, K. and Major, S. (1980) Studies on the mechanism of action of anti-tumor-promoting agents: their specificity in two-stage promotion. Proc. Natl. Acad. Sci USA 77: 2251–2254.

Smirnoff, P., Khalef, S., Birk, Y. and Applebaum, S. W. (1979) Trypsin and chymotrypsin inhibitor from chick peas. Int. J. Pept. Protein Res. 14: 186–192.

Solomon, D. H., O'Brian, C. A. and Weinstein, I. B. (1985) N-α-tosyl-L-lysine chloromethyl ketone and N-α-tosyl-L-phenylalanine chloromethyl ketone inhibit protein kinase C. FEBS Lett. 190: 342–344.

Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F. and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6075–6086.

Soy Protein Council (1987) Soy Protein Products: Characteristics, Nutritional Aspects and Utilization, pp. 7–13. Washington, DC.

Spangler, W. L., Gumbmann, M. R., Liener, I. E. and Rackis, J. J. (1985) The USDA Trypsin Inhibitor Study III. Sequential

development of pancreatic pathology in rats. Qual. Plant Foods Hum. Nutr. 35: 259–274.

St. Clair, W. H. (1991) Suppression of 3-methylcholanthrene-induced cellular transformation by timed administration of the Bowman-Birk protease inhibitor. Carcinogenesis 12: 935–937.

St. Clair, W. H. and St. Clair, D. K. (1991) Effect of the Bowman-Birk protease inhibitor on the expression of oncogenes in the irradiated rat colon. Cancer Res. 51: 4539–4543.

St. Clair, W., Billings, P., Carew, J., Keller-McGandy, C., Newbene, P. and Kennedy, A. R. (1990a) Suppression of DMH-induced carcinogenesis in mice by dietary addition of the Bowman-Birk protease inhibitor. Cancer Res. 50: 580–586.

St. Clair, W. H., Billings, P. C. and Kennedy, A. R. (1990b) The effects of the Bowman-Birk protease inhibitor on c-myc expression and cell proliferation in the unirradiated and irradiated mouse colon. Cancer Lett. 52: 145–152.

St. Clair, W. H., St. Clair, D. K. and Kennedy, A. R. (1991) Comparison of the Bowman-Birk protease inhibitor to WR1065 for protection against radiation-induced DNA, cellular and tissue damage. Cancer J. 4: 278–282.

Strickland, J. E., Greenhalgh, D. A., Koceva-Chyla, A., Hennings, H., Restrepo, C., Balaschak, M. and Yuspa, S. H. (1988) Development of murine epidermal cell lines which contain an activated ras[Ha] oncogene and form papillomas in skin grafts on athymic nude mice host. Cancer Res. 48: 165–169.

Struthers, B. J., MacDonald, J. R., Dahlgren, R. R. and Hopkins, D. T. (1983) Effects on the monkey, pig and rat pancreas of soy products with varying levels of trypsin inhibitor and comparison with the administration of cholecystokinin. J. Nutr. 113: 86–97.

Su, L. N., Toscano, W. A., Jr. and Kennedy, A. R. (1991) Suppression of phorbol ester-enhanced radiation-induced malignancy in vitro by protease inhibitors is independent of protein kinase C. Biochem. Biophys. Res. Commun. 176: 18–24.

Su, Z. Z., Yemul, S., Stein, C. A. and Fisher, P. B. (1995) c-fos is a positive regulator of carcinogen enhancement of adenovirus transformation. Oncogene 10: 2037–2049.

Sugita, H., Ishiura, S., Suzuki, K. and Imahori, K. (1980) Inhibition of epoxide derivatives on chicken calcium-activated neutral protease (CANP) in vitro and in vivo. J. Biochem. 87: 339–341.

Swartz, L. B. and Austen, K. F. (1984) Structure and function of the chemical mediators of mast cells. Prog. Allergy 34: 271–321.

Tait, L., Soule, H. D. and Russo, J. (1990) Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6087–6094.

Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. 1. Purification and characterization of an active enzyme from bovine cerebellum. J. Biol. Chem. 252: 7603–7609.

Takai, Y., Kishimoto, A., Iwasa, M., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipid. J. Biol. Chem. 254: 3692–3695.

Takechi, H., Hirayoshi, K., Nakai, A., Kudo, H., Saga, S. and Nagata, K. (1992) Molecular cloning of a mouse 47-kDa heat-shock protein (HSP47), collagen-binding stress protein, and its expression during the differentiation of F9 teratocarcinoma cells. Eur. J. Biochem. 206: 323–329.

Taylor, W. R., Egan, S. E., Mowat, M., Greenberg, A. H. and Wright, J. A. (1992) Evidence for synergistic interactions

between ras, myc and a mutant form of p53 in cellular transformation and tumor dissemination. Oncogene 7: 1383–1390.

Terzaghi-Howe, M. (1989) Induction of preneoplastic alterations by X-rays and neutrons in exposed rat tracheas and isolated tracheal epithelial cells. Radiat. Res. 120: 352–363.

Tlsty, T. O., Brown, P. C. and Schimke, R. T. (1984) Ultraviolet radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells. Mol. Cell. Biol. 4: 1050–1056.

Tominaga, S. (1985) Cancer incidence in Japanese in Japan, Hawaii, and Western United States. Natl. Cancer Inst. Monogr. 69: 83–92.

Torun, B., Pineda, O., Vireti, F. E. and Arrayave, G. (1982) Use of amino acid composition data to predict protein nutritive value for children with specific reference to new estimates for their essential amino acid requirements. In: Protein Quality in Humans: Assessment and In Vitro Evaluation, pp. 374–393, Bodwell, C. E., Adkins, J. S. and Hopkins, D. T. (eds.) AVI Publishing Co., Westport.

Toskes, P. P. (1986) Negative feedback inhibition of pancreatic exocrine secretion in humans. Adv. Exp. Med. Biol. 199: 142–152.

Travis, J. and Salvesen, G. S. (1983) Human plasma proteinase inhibitors. Annu. Rev. Biochem. 52: 655–709.

Travis, J., Bowen, J. and Baugh, R. (1978) Human α-1-antichymotrypsin: interaction with chymotrypsin-like proteinases. Biochemistry 17: 5651–5656.

Troll, W. (1976) Blocking tumor promotion by protease inhibitors. In: Fundamentals in Cancer Prevention, pp. 41–55, Magee, P. M., Takayama, S., Sugimura, T. and Matsushima, T. (eds.) University Park Press, Baltimore.

Troll, W. and Kennedy, A. R. (eds.) (1993) Protease Inhibitors as Cancer Chemopreventive Agents. Plenum Publishing Corporation, New York and London.

Troll, W., Klassen, A. and Janoff, A. (1970) Tumorigenesis in mouse skin: inhibition by synthetic inhibitors of proteases. Science 169: 1211–1213.

Troll, W., Belman, S., Wiesner, R. and Shellabarger, C. J. (1979a) Protease action in carcinogenesis. In: Biological Functions of Proteinases, pp. 165–170, Holtzer, H. and Tschesche, H. (eds.) Springer-Verlag, Berlin, Heidelberg, New York.

Troll, W., Weisner, R., Belman, S. and Shellabarger, C. J. (1979b) Inhibition of carcinogenesis by feeding diets containing soybeans. Proc. Am. Assoc. Cancer Res. 20: 265.

Troll, W., Weisner, R., Shellabarger, C. J., Holtzman, S. and Stone, J. P. (1980) Soybean diet lowers breast tumor incidence in irradiated rats. Carcinogenesis 1: 469–472.

Troll, W., Witz, G., Goldstein, B., Stone, D. and Sugimura, T. (1982) The role of free oxygen radicals in tumor promotion and carcinogenesis. In: Carcinogenesis: A Comprehensive Survey; Cocarcinogenesis and Biological Effects of Tumor Promoters, Vol. 7, pp. 593–597, Hecker, E., Fusenig, N. E., Kunz, W., Marks, F. and Theilmann, H. W. (eds.) Raven Press, New York.

Troll, W., Frenkel, K. and Wiesner, R. (1984) Protease inhibitors as anticarcinogens. J. Natl. Cancer Inst. 73: 1245–1250.

Umans, R. S., Weichselbaum, R. R., Johnson, C. M. and Kennedy, A. R. (1984) Protease inhibitor antipain reduces nuclear binding of estrogen-receptor complex in MCF-7 breast tumor cells. Carcinogenesis 5: 1355–1357.

Umans, R. S., Radner, B. and Kennedy, A. R. (1985) Effects of protease inhibitors on nuclear binding of glucocorticoid hormones in C3H10T1/2 cells. Fed. Eur. Biochem. Soc. 193: 27–30.

Umezawa, K. (1972) Enzyme Inhibitors of Microbial Origin. University of Tokyo Press, Tokyo.

Verhey, L. J. and Sedlacek, R. (1983) Determination of the radioprotective effects of topical applications of MEA, WR2721 and N-acetylcysteine on murine skin. Radiat. Res. 93: 175–183.

von Hofe, E., Brent, R. and Kennedy, A. R. (1990) Inhibition of X-ray induced exencephaly by protease inhibitors. Radiat. Res. 108–111.

von Hofe, E., Newberne, P. M. and Kennedy, A. R. (1991) Inhibition of N-nitrosomethylbenzylamine induced esophageal neoplasms by the Bowman-Birk protease inhibitor. Carcinogenesis 12: 2147–2150.

Wan, X. S., Koch, C. J., Lord, E. M., Manzone, H., Billings, P. C., Donahue, J. J., Odell, C. S., Miller J. and Kennedy, A. R. (1995) Monoclonal antibodies differentially reactive with native and reductively modified Bowman-Birk protease inhibitor. J. Immunol. Methods 180: 117–130.

Ware, J. H., Wan, X. S., Rubin, H., Schechter, N. M. and Kennedy, A. R. (1997) Soybean Bowman-Birk protease inhibitor is a highly effective inhibitor of human mast cell chymase. Arch. Biochem. Biophys. 344: 133–138.

Wargovich, M. J., Bauer, A. R., Hu, P. J. and Sumiyoshi, H. (1988) Dietary factors and colorectal cancer. Gastroenterol. Clin. North Am. 7: 727–745.

Watanabe, S., Shiratori, K., Takeuchi, T. and Chey, W. Y. (1986) Intrajejunal administration of a synthetic trypsin inhibitor (camostate) stimulates the release of endogenous secretin but not cholecystokinin in humans. Gastroenterology 90: 1685.

Weed, H., McGandy, R. B. and Kennedy, A. R. (1985) Protection against dimethylhydrazine induced adenomatous tumors of the mouse colon by the dietary addition of an extract of soybeans containing the Bowman-Birk protease inhibitor. Carcinogenesis 6: 1239–1241.

Weeke, B. and Jarnum, S. (1971) Serum concentrations of 19 serum proteins in Crohn's disease and ulcerative colitis. Gut 12: 297–302.

Weinstein, I. B. (1978) Current concepts on mechanisms of chemical carcinogenesis. Bull. NY Acad. Med. 54: 366–383.

Werner, M. H. and Wemmer, D. E. (1992) Three-dimensional structure of soybean trypsin/chymotrypsin Bowman-Birk Inhibitor in solution. Biochemistry 31: 999–1010.

Winn, D. M., Ziegler, R. G., Pickle, L. W., Gridley, G., Blot, W. J. and Hoover, R. N. (1984) Diet in the etiology of oral and pharyngeal cancer among women from the southern United States. Cancer Res. 44: 1216–1222.

Wintersberger, U. (1984) The selective advantage of cancer cells: a consequence of genome mobilization in the course of the induction of DNA repair processes? (model studies on yeast). Adv. Enzyme Regul. 22: 311–323.

Witkin, E. M. (1976) Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol. Rev. 40: 869–907.

Witschi, H. and Kennedy, A. R. (1989) Modulation of lung tumor development in mice with the soybean-derived Bowman-Birk protease inhibitor. Carcinogenesis 10: 2275–2277.

Witte, J. (1979) Endotoxinamie und hyperdynamer septischer Schock: Pathobiochemie ausgewahlter Gerinnungs- und anderer Plasma-Protein-parameter. Habilitation script, Medical Faculty of the University of Munich.

Witz, G., Goldstein, B. D., Amoruso, M., Stone, D. S. and Troll, W. (1980) Retinoid inhibition of superoxide anion radical production by human polymorphonuclear leukocytes stimulated with tumor promoters. Biochem. Biophys. Res. Commun. 97: 883–888.

Wolf, W. J. and Cowan, J. C. (1975) Soybeans as a Food Source. CRC Press, Inc., Cleveland.

Wynder, E. L. (1975) An epidemiological evaluation of the causes of cancer of the pancreas. Cancer Res. 35: 2228–2233.

Yamamoto, R. S., Umezawa, H., Takeuchi, T., Matsushima, T., Hara, K. and Sugimura, T. (1974) Effect of leupeptin on colon carcinogenesis in rats with azoxymethane. Proc. Am. Assoc. Cancer Res. 15: 38.

Yamamura, M., Nakamura, N., Fukui, Y., Takamura, C., Yamamoto, M., Minato, Y., Tamura, Y. and Jujii, S. (1978) Inhibition of 7,12 DMBA-induced mammary tumorigenesis by a synthetic protease inhibitor, N,N-dimethylamino (p-p'-guanidino-benzoyloxy) benzilcarbonyloxyglycolate. Gann 69: 749–752.

Yamauchi, Y., Kobayashi, T. and Watanabe, A. (1987) Anticarcinogenic effects of a serine protease inhibitor (FOY-305) through the suppression of neutral serine protease activity during chemical hepatocarcinogenesis in rats. Hiroshima J. Med. Sci. 36: 81–87.

Yanatori, Y. and Fujita, T. (1976) Hypertrophy and hyperplasia in the endocrine and exocrine pancreas of rats fed soybean trypsin inhibitor or repeatedly injected with pancreozymin. Arch. Histol. Japan 39: 67–78.

Yavelow, J., Gidlund, M. and Troll, W. (1982) Protease inhibitors from processed legumes effectively inhibit superoxide generation in response to TPA. Carcinogenesis 1: 135–138.

Yavelow, J., Finlay, T. H., Kennedy, A. R. and Troll, W. (1983) Bowman-Birk soybean protease inhibitor as an anticarcinogen. Cancer Res. 43: 2454–2459.

Yavelow, J., Collins, M., Birk, Y., Troll, W. and Kennedy, A. R. (1985) Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress X-ray induced transformation in vitro. Proc. Natl. Acad. Sci. USA 82: 5395–5399.

Yavelow, J., Caggaga, M. and Beck, K. A. (1987) Proteases occurring in the cell membrane: a possible receptor for the Bowman-Birk type of protease inhibitors. Cancer Res. 47: 1598–1601.

Yavelow, J., Schepis, L. T., Nickels, J. and Ritchie, G. (1993) Cell membrane enzymes containing chymotrypsin-like activity. In: Protease Inhibitors as Cancer Chemopreventive Agents, pp. 217–226, Troll, W. and Kennedy, A. R. (eds.) Plenum Publishing Corp., New York and London.

Yen, J. T., Jensen, A. H. and Simon, J. (1977) Effect of dietary raw soybean and soybean trypsin inhibitor on trypsin and chymotrypsin activities in the pancreas and in small intestinal juice of growing swine. J. Nutr. 107: 156–165.

Young, V. R., Wayler, A., Garza, C., Steinke, F. H., Murray, E., Rand, W. M. and Scrimshaw, N. S. (1984) A long-term metabolic balance study in young men to assess the nutritional quality of an isolated soy protein and beef proteins. Am. J. Clin. Nutr. 39: 8–15.

Yuspa, S. H., Koehler, B. A., Kulesz-Martin, M. and Hennings, H. (1981) Clonal growth of mouse epidermal cells in medium with reduced calcium concentration. J Invest. Dermatol 76: 144–146.

Zawaski, K., Gruebele, A., Kaplan, D., Reddy, S., Mortensen, A. and Novak, R. F. (1993) Evidence for enhanced expression of c-fos, c-jun, and the Ca^{2+}-activated neutral protease in rat liver following carbon tetrachloride administration. Biochem. Biophys. Res. Commun. 197: 585–590.